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The goals of this project are to construct single-chain bispecific antibodies ($scFv_2$) that target erbB-2 on breast cancer cells, to evaluate the susceptibility of the tumor cells to lysis mediated by bispecific antibodies, and to develop an animal model that can evaluate the *in vivo* effectiveness of these agents. The following aims were accomplished during the past year: 1) Two different $scFv_2$ were constructed, purified, and tested *in vitro*. Both antibodies contained the anti-erbB-2 scFv, 800E6. One of the antibodies contained the anti-T cell receptor scFv 1B2 and the other contained the anti-Vβ scFv KJ16. Although both $scFv_2$ bound to the appropriate antigens, current efforts are devoted to increasing the fraction of the $scFv_2$ that have activity. 2) Transgenic mice (called TCR/RAG$^{-/-}$) that contain a single population of cytotoxic T cells were fully characterized. The mice expressed >90% CD8+ peripheral T cells, which could be activated *in vitro* to lyse the appropriate target cells. The TCR/RAG$^{-/-}$ mice also accepted xenografts of two erbB-2^{+} human tumor cell lines, BT474 and SKOV3. Future work will involve strategies to activate T cells *in vivo*, so that the optimal conditions for tumor targeting can be explored.

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David M. Kranz 9/1/96

PI - Signature Date

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INTRODUCTION
(Adapted in part from original proposal)

Successful treatment of breast cancer requires the identification of specific targets for the rational design of therapeutic agents. One such target, described several years ago, is the oncogene product erbB-2 (1-3). This protein is expressed on the surface of tumor cells in approximately 30% of women with the poorest prognosis for survival (4). The expression of erbB-2 does not appear to be correlated, either positively or negatively, with the estrogen receptor (5), although a recent report suggests that the erbB-2 signalling pathway targets the estrogen receptor resulting in estrogen-independent growth of tumor cells (6). However, it's expression is associated with an increase in the resistance of tumor cells to lysis by some natural immune mechanisms such as tumor necrosis factor and lymphokine activated killer cells (7,8).

A number of investigators have now begun to use antibodies specific for erbB-2 as possible therapeutic agents (9-16). The agents include immunotoxins, radioimmunoconjugates, and bispecific antibodies. The latter antibodies are intended to mediate effects by directing the lysis of tumor cells through cytotoxic effector cells. One of the predicted advantages of bispecific antibodies is that they should not have side effects associated with delivery of toxins or isotopes. Bispecific antibodies have in fact begun to be examined in several clinical trials, with some encouraging results.

Despite their promise and emergence into clinical trials, there are many questions that need to be addressed before optimal uses, with minimal side effects, of bispecific antibodies can be realized. For example, genetic engineering now provides a method for constructing smaller, potentially more stable, antibodies. Are these antibodies likely to be more effective than conventional intact antibodies? Will the natural resistance of erbB-2^+ tumors cells present an obstacle to successful bispecific antibody therapy? Can a system that uses a patients own immune cells be developed so that *ex vivo* activation of effector cells is not needed? This would obviously allow the treatment of a much larger patient base than would be possible if effector cells must be cultured for every patient.

It would clearly be useful to have a system that could provide answers to these questions in order to design the most effective clinical trials of bispecific antibodies. There has been no animal model developed that can evaluate all of these issues using human breast cancer cells. The purpose of this project is twofold. First, to construct novel bispecific antibodies that can be expressed as a single-chain in *E. coli* (17). Second, to develop an *in vivo* animal model that will allow the testing of these agents in comparison with other conventional bispecific antibodies. There are many potential therapeutic regimens that will need to be evaluated. To do so, an animal model that will not require introduction of human effector cells and that will most resemble the situation that will be encountered in the human disease will be developed. It is anticipated that these animals could also serve as models for immune modulating agents that are developed by other laboratories.

The methods used to approach these goals involve many reagents and strains of mice that have been developed in my lab and in collaboration with others over the past ten years. The general strategy involves the use of the following: 1) Monoclonal antibodies and single-chain antibodies that are specific for the T cell receptor of a mouse cytotoxic T lymphocyte clone 2C (18); 2) Monoclonal antibodies that are specific for human erbB-2, provided by Pier Natali (11); 3) Cloning and expression systems for the overproduction of single-chain monospecific and bispecific antibodies (17,18); 4) Human breast cancer tumor cell lines obtained from the ATCC; 5) Transgenic mice that express the TCR from CTL clone 2C (19,20); and 6) RAG-1 knockout mice, provided by Susumu Tonegawa's lab, that lack B and T cells because of the absence of recombinational-activation-gene 1 (21).

The four specific aims of the project are: 1) Following a strategy that has recently proven successful in our lab, to engineer a single-chain bispecific antibody to erbB-2 and the TCR of a mouse CTL clone; 2) To use a simple screening method to search for agents that increase the sensitivity of erbB-2^+ breast cancer cell lines to lysis by CTL. 3) To develop an *in vivo* model for targeting transplanted human breast cancer cells using immunodeficient, TCR transgenic mice (TCR/recombination-activating-gene knockouts, RAG-2^-). 4) To test the *in vivo* effectiveness of various bispecific antibody regimens in the TCR/RAG-2^- human xenograft system. Specific details concerning progress in the second year of this award toward these goals are provided in the body of this report, below.

BODY

Additional progress has been made toward various aspects of specific aims 1 and 3. Methods, results, and a brief discussion are presented within each aim:

Specific Aim 1. Construct and characterize a single-chain bispecific antibody (anti-erbB-2/anti-TCR, called $scFv_2$).

In last year's report, the cloning, expression, and characterization of the V_H and V_L genes from the anti-erbB-2 antibody 800E6 was described. This single-chain gene has now been cloned downstream of the anti-TCR antibodies 1B2 and KJ16 to produce two different $scFv_2$. 1B2 recognizes a clonotypic determinant on the TCR from CTL clone 2C (22) while KJ16 recognizes the Vβ8 chain of clone 2C (23). Both $scFv_2$ contained the 25 residue interchain linker 205 and a 10 residue c-*myc* tail for detection (Figure 1). These two antibodies were constructed in order to evaluate the effect of different antibody domains on folding of the $scFv_2$ and ultimately on their bispecific activity.

Construction and Expression of $scFv_2$. To construct the $scFv_2$, the 800E6 scFv was amplified by PCR using a 5' primer which contained the sequence for the 205c' linker, and a 3' primer that included a HindIII site within the c-*myc* region for cloning into the 1B2 scFv vector or the KJ16 scFv vector. The linker/800E6 scFv fragment was cloned between the $1B2V_H$ (or KJ16 V_H) and the *c-myc* tail. Inclusion body pellets from cells that contained the $scFv_2$ genes were solubilized in 6M guanidine. To isolate monomeric $scFv_2$ from proteins which remained aggregated in the presence of guanidine, the solubilized inclusion body was subjected to Superdex

G-200 size exclusion in 4M guanidine. The 60 kDa peak fractions were individually refolded by dialysis into Tris-Arginine buffer, and analyzed by SDS-PAGE (data not shown). Fractions with the highest fraction of homogeneous $scFv_2$ were pooled, dialysed against buffer and monomeric $scFv_2$ was isolated by non-denaturing size exclusion chromatography (Figure 2). The peak that represented monomer had a 60kD apparent molecular weight under reducing and non-reducing conditions (data not shown). As expected, this was approximately twice the size of either the 1B2, KJ16, or 800E6 scFv (30kD, Figure 2).

Figure 1. Schematic of $scFv_2$ gene for single-chain bispecific anti-TCR/anti-erbB-2 antibodies.

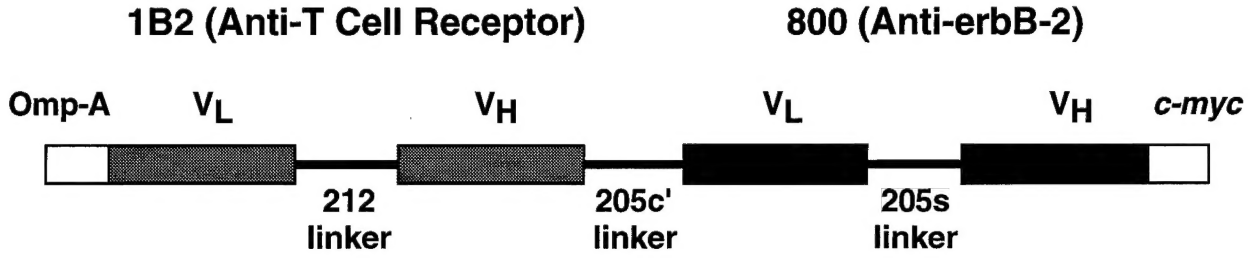
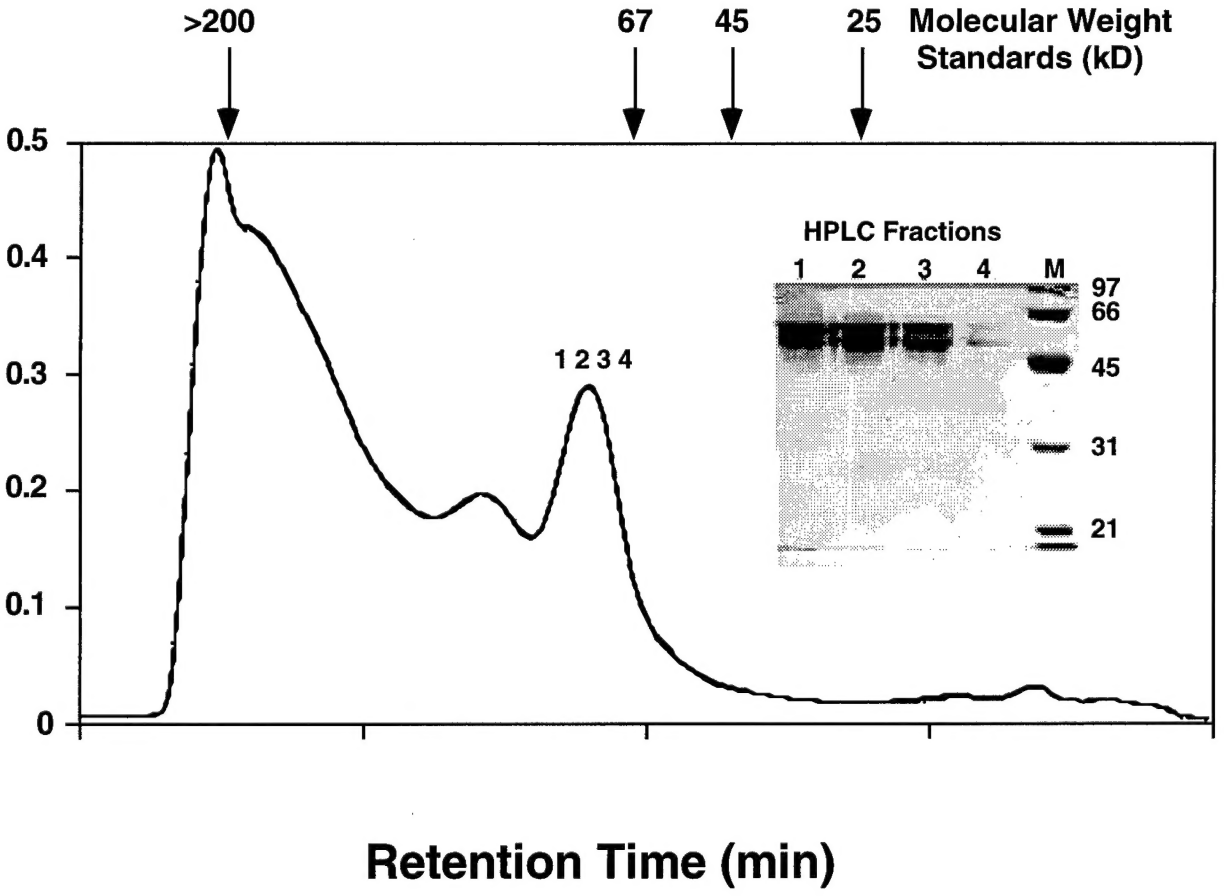
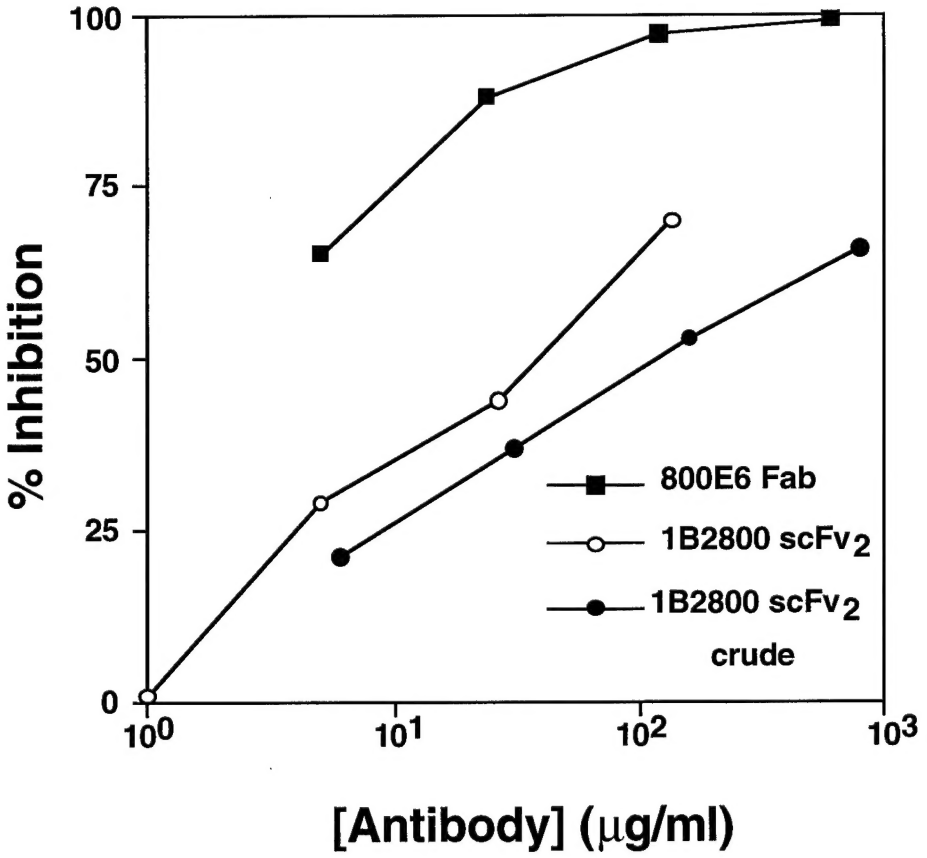


Figure 2. Gel filtration (G200) profile and SDS-PAGE analysis of 1B2/800E6 $scFv_2$ protein.



Binding Properties of Anti-TCR/erbB-2 scFv$_2$ Antibodies. To examine binding of the scFv$_2$ to erbB-2 on the surface of human breast cancer cells, a competition assay was performed with ^{125}I-labeled 800E6 Fab fragments. As shown in Figure 3, crude as well as partially purified preparations of 1B2 scFv$_2$ were able to bind erbB-2 on SKBR-3 cells. In contrast to the monovalent 800E6 scFv which bound erbB-2 at nearly equimolar concentrations as 800E6 Fab fragments, bispecific antibody preparations were 10 to 25-fold less efficient than unlabeled Fab fragments. A possible explanation for the observed reduction in binding is that a significant fraction of the scFv$_2$ preparation is not properly folded.

Figure 3. Binding of 1B2/800E6 scFv$_2$ erbB2 on the human tumor line SKBR-3. HPLC purified scFv$_2$ or crude scFv$_2$ were compared with unlabeled Fab fragments for their ability to inhibit binding of ^{125}I-labeled 800E6 Fab fragments.



The KJ16/800E6 scFv$_2$ was also examined in an ELISA for binding to soluble T cell receptor absorbed to 96-well plates (Figure 4). This antibody (but not the 1B2 scFv$_2$, data not shown) was found to bind specifically to the soluble TCR and to the cell surface of CTL 2C by flow cytometry (data not shown). At this time it is unclear why detectable binding of the 1B2 scFv$_2$ was not detected, although it is likely due to the less efficient folding of this scFv domain compared to the KJ16 scFv domain. In addition, the KJ16/800E6 scFv$_2$ bound to erbB-2+ tumor cell line, BT474 as judged by flow cytometry (Figure 5).

Figure 4. Binding of KJ16/800E6 $scFv_2$ to soluble 2C scTCR. ELISA results using soluble TCR adsorbed to 96 well plates, followed by various dilutions of the $scFv_2$. Binding was detected with a monoclonal antibody to c-myc followed by HRP-labeled anti-mouse IgG.

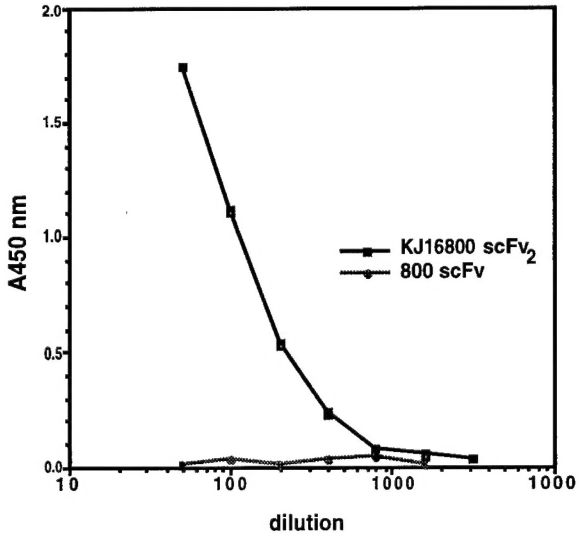
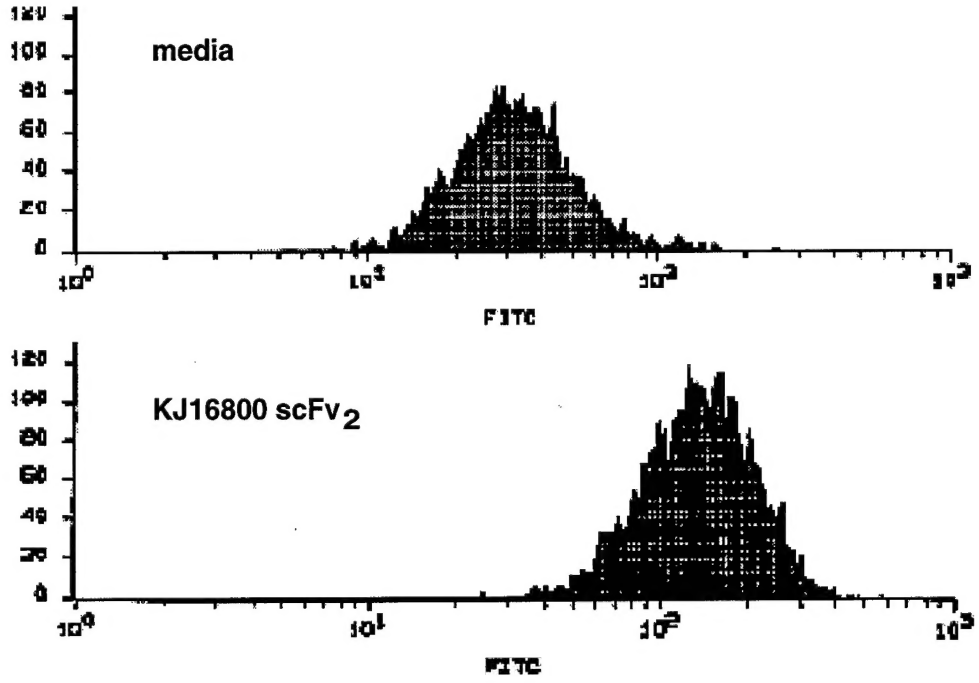


Figure 5. Binding of KJ16/800E6 $scFv_2$ to the tumor line BT-474. Flow cytometry of the erbB-2^+ tumor BT474 incubated with either media or $scFv_2$, followed by a mouse monoclonal antibody to c-myc and fluorescein-labeled anti-mouse IgG.



Bispecific Antibody Activity in CTL-Mediated Killing Assays. As the $scFv_2$ had erbB-2 binding activity, it was of interest to examine if the antibodies could redirect CTL 2C to lyse target cells. At this time we have only tested the 1B2 $scFv_2$ but will soon complete studies with KJ16 $scFv_2$. This was of particular interest because no one has yet reported redirected killing with an anti-erbB-2 $scFv_2$ and it was possible that this property could be influenced by the nature of the inter-scFv linker. As expected, the L^d alloreactive CTL 2C was unable to lyse human, erbB-2 positive breast carcinoma line BT-474 without the bispecific antibody. However, BT-474 were lysed when 1B2/800E6 $scFv_2$ was added to the CTL/target cell assay (Table 1). The observed lysis was dependent on the concentration of bispecific antibody (Table 1, Exp. 2), and could be inhibited with monospecific 1B2 or 800 intact Ab (Table 1, Exp. 1). The ability to mediate lysis requires covalent linkage of the two antibody binding domains as evidenced by the inability of a mixture of 1B2 and 800E6 monospecific antibodies to redirect lysis (Table 1, Exp.1).

Table 1. Redirected lysis of erbB-2+ tumor cells by the 1B2/800E6 $scFv_2$.

Experiment 1	antibody	µg/ml	Inhibitor	% Specific ^{51}Cr Release BT-474
	-	-	-	0
	1B2 Ig + 800E6 Ig	25 + 25	-	4
	$scFv_2$	13	-	22
	$scFv_2$	13	25 µg/ml 1B2 Ig	0
	$scFv_2$	13	25 µg/ml 800E6 Ig	0
	$scFv_2$	13	-	0*

Experiment 2	antibody	µg/ml	% Specific ^{51}Cr Release BT-474
	$scFv_2$	5	20
	$scFv_2$	2.5	21
	$scFv_2$	1.3	18
	$scFv_2$	0.6	6

*In this assay, target cells were added to wells with media and $scFv_2$, without effector cells. In all other wells 2C CTLs were added at an effector to target cell ratio of 10:1. All assays were performed in triplicate and standard error was less than ± 3%.

The observation that the 1B2 $scFv_2$ could redirect lysis was encouraging in light of the finding that both the anti-TCR and anti-erbB2 domains appear to be only partially folded (<5% by our estimates). Thus, the relatively high concentrations of $scFv_2$ required for activity (>1 ug/ml) might reasonably expect to be reduced if we can obtain fully folded forms of the $scFv_2$. In addition, the KJ16 $scFv_2$ may be more effeciently folded based on the anti-TCR results described above. Our future efforts toward each of these aims are described below.

Specific Aim 3. Develop an *in vivo* model for targeting transplanted human breast cancer cells using immunodeficient, T cell receptor transgenic mice.

The breeding, maintenance, and testing of immunodeficient, T cell receptor transgenic mice was described in last year's report. A continuous colony of $TCR^{+/+}RAG^{-/-}$ mice that express a monoclonal population of $CD8^+$ T cells with the TCR from CTL clone 2C has now been established. Peripheral lymphocytes (blood and spleen) and thymocytes from $TCR^{+/+}RAG^{-/-}$ have been analyzed by flow cytometry in order to characterize their CD4, CD8 and TCR phenotypes. In addition, splenocytes have been cultured *in vitro* to show that they can be activated to produce a cytolytic population. Finally, two human tumor cell lines have now been successfully transplanted into the $TCR^{+/+}RAG^{-/-}$ mice. Each of these studies are described below.

Phenotype of Peripheral Lymphocytes and Thymocytes from $TCR^{+/+}RAG^{-/-}$ mice.

The affinity of the TCR for MHC class I or II is thought to direct the effector fate of the T cell toward a $CD4^+$ helper or $CD8^+$ cytotoxic T cell phenotype. Since the $TCR/RAG^{-/-}$ mice express only the 2C TCR, lymphocyte subsets were examined to determine if a monoclonal population of T cells would express only CD8 (like the 2C CTL clone). Spleen, thymus, and blood samples were analyzed for CD4, CD8, Thy1.2, and 2C TCR using mutiple color flow cytometry (Figure 6, blood data not shown). Males and females ages 1-6 months were examined, with very similar results at all ages (data not shown). $TCR/RAG^{-/-}$ thymocytes were skewed toward a $CD8^+4^-$ population. In contrast to C57Bl/6 normal mice in which >95% thymocytes are $CD4^+8^+$, $TCR/RAG^{-/-}$ thymocytes were on average 49% $CD8^+4^-$, 45% $CD4^+8^+$, and 5% $CD4^+8^-$ (Figure 6).

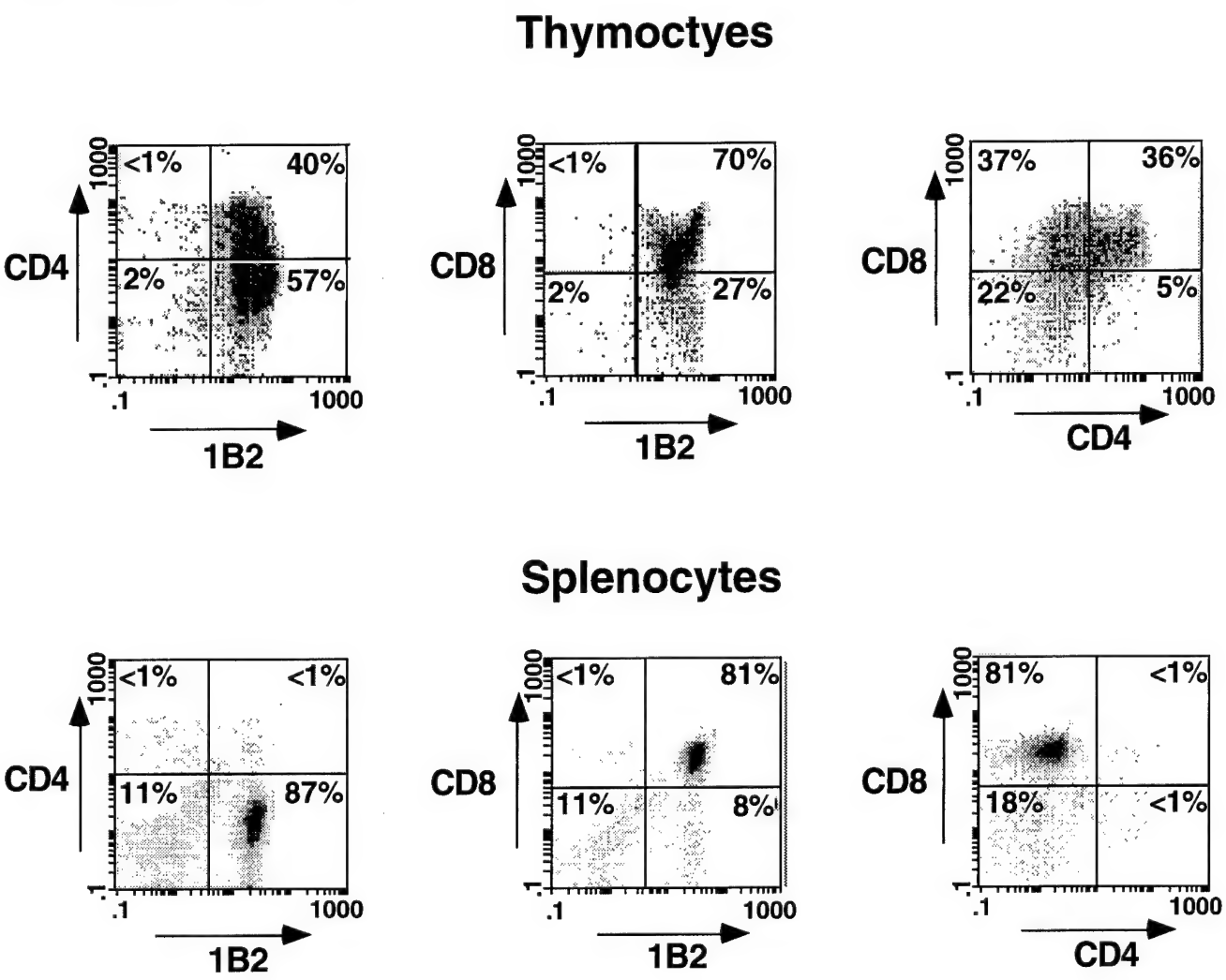
The skewing toward a $CD8^+$ phenotype was dramatic in the peripheral T cells of the $TCR/RAG^{-/-}$ mouse where >98% were $CD8^+4^-$ (Figure 6). This is in contrast to C57Bl/6 normal mice which had 60% $CD4^+8^-$ and 40% $CD8^+4^-$. Although splenocytes from 2C TCR transgenic mice were also skewed toward $CD8^+4^-$ (~70%), there were a significant number of $CD4^+CD8^-$ T cells (~30%) in the periphery (Gruber and Kranz, unpublished results; ref 19). In contrast, the $TCR/RAG^{-/-}$ mice expressed very low to undetectable levels of $CD4^+8^-$ T cells (0-5%), with nearly all T cells being $1B2^+CD8^+4^-$ (93-99%) (Figure 6).

***In Vitro* Activation of Peripheral Lymphocytes from $TCR^{+/+}RAG^{-/-}$ mice.**

Most peripheral T cells exist in a resting state in which they can not secrete cytokines or lyse target cells. Even though the $TCR/RAG^{-/-}$ mice displayed a monoclonal population of $CD8^+$ T cells, as might have been anticipated, these CTLs must have the ability to be activated, proliferate, and lyse target cells in order to be useful in immunotherapy.

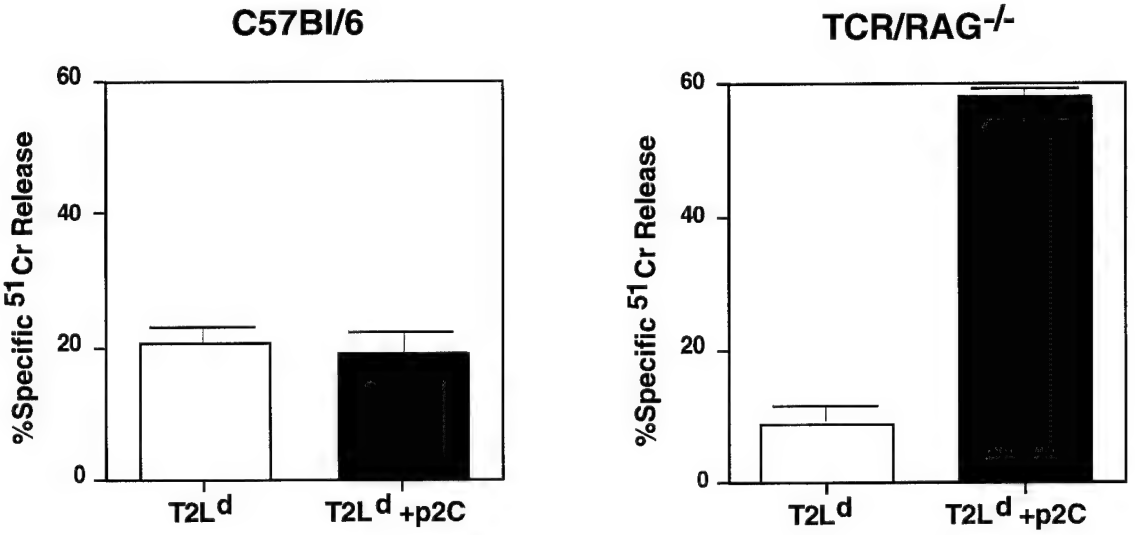
CTL activation was examined by incubation of the $TCR/RAG^{-/-}$ splenocytes with the superantigen, staphylococcal enterotoxin B (SEB). SEB has been used in many studies as an *in vitro* and *in vivo* T cell activating agent, and was therefore of interest for use with the $TCR/RAG^{-/-}$ mice. SEB has been shown to bind the β chain of TCRs that contain a Vβ8 TCR gene product (23). SEB binds to the TCR and to a MHC class II molecule, which allows activation of the CTL regardless of peptide/MHC class I specificity (24). Although ~20% of T cells in a normal mouse will express Vβ8 TCRs, all of the CTLs from the $TCR/RAG^{-/-}$ mouse express Vβ8.2 and thus should be responsive to SEB activation, if sufficient IL-2 is present as a growth factor.

Figure 6. Flow cytometric analysis of thymic and splenic lymphocyte subsets from a TCR/RAG$^{-/-}$ mouse. 1B2 is a monoclonal antibody that is specific only for the TCR derived from CTL 2C. 1B2 was directly labeled with fluorescein isothiocyanate; anti-CD4 was labeled with phycoerythrin; anti-CD8 was labeled with cychrome.



To test for the specific activation of 2C TCR^{+} CTLs from the TCR/RAG$^{-/-}$ spleen, a chromium release assay was performed with the activated splenocytes. The target cell was a human cell line transfected with MHC class I haplotype, L^d, called $T2L^d$. This cell line expresses L^d from the tranfected gene, but does not possess the mouse peptide, p2C (25). Incubation of $T2L^d$ with the peptide, p2C will "load" the L^d MHC, providing the alloantigen which is recognized by the 2C TCR. $T2L^d$ without the peptide serves as a control for 2C TCR specific lysis as it shows the amount of lysis from cells which lyse independent of $p2C/L^d$. SEB activated $p2C/L^d$-specific CTLs that were able to lyse $T2L^d$ only after addition of the peptide, p2C (Figure 7). As mentioned, ~20% of C57Bl/6 T cells would be expected to respond to SEB activation. However, very few of these CTLs express a TCR that is specific for the $p2C/L^d$ complex. A moderate amount of lysis was observed for $T2L^d$ with and without the peptide, p2C. This non-specific lysis may be due to the induction of cytolytic cells that have no MHC/peptide restriction or that recognize L^d without the specific peptide, p2C.

Figure 7. SEB mediated activation of CTLs from TCR+/+RAG-/- mice. Spleen cells from a normal C57Bl/6 mouse and a TCR/RAG mouse were stimulated for 3 days *in vitro* and assayed for cytotoxicity using the target cell for the 2C CTL clone, $T2L^d$ and the peptide p2C.



In conclusion, the TCR/RAG-/- mice express a monoclonal population of $CD8^+$ T cells that are capable of being activated *in vitro*. These activated T cells lyse target cells that express only the specific alloantigen ($p2C/L^d$).

Xenografts of Human Tumors in $TCR^{+/+}RAG^{-/-}$ mice.

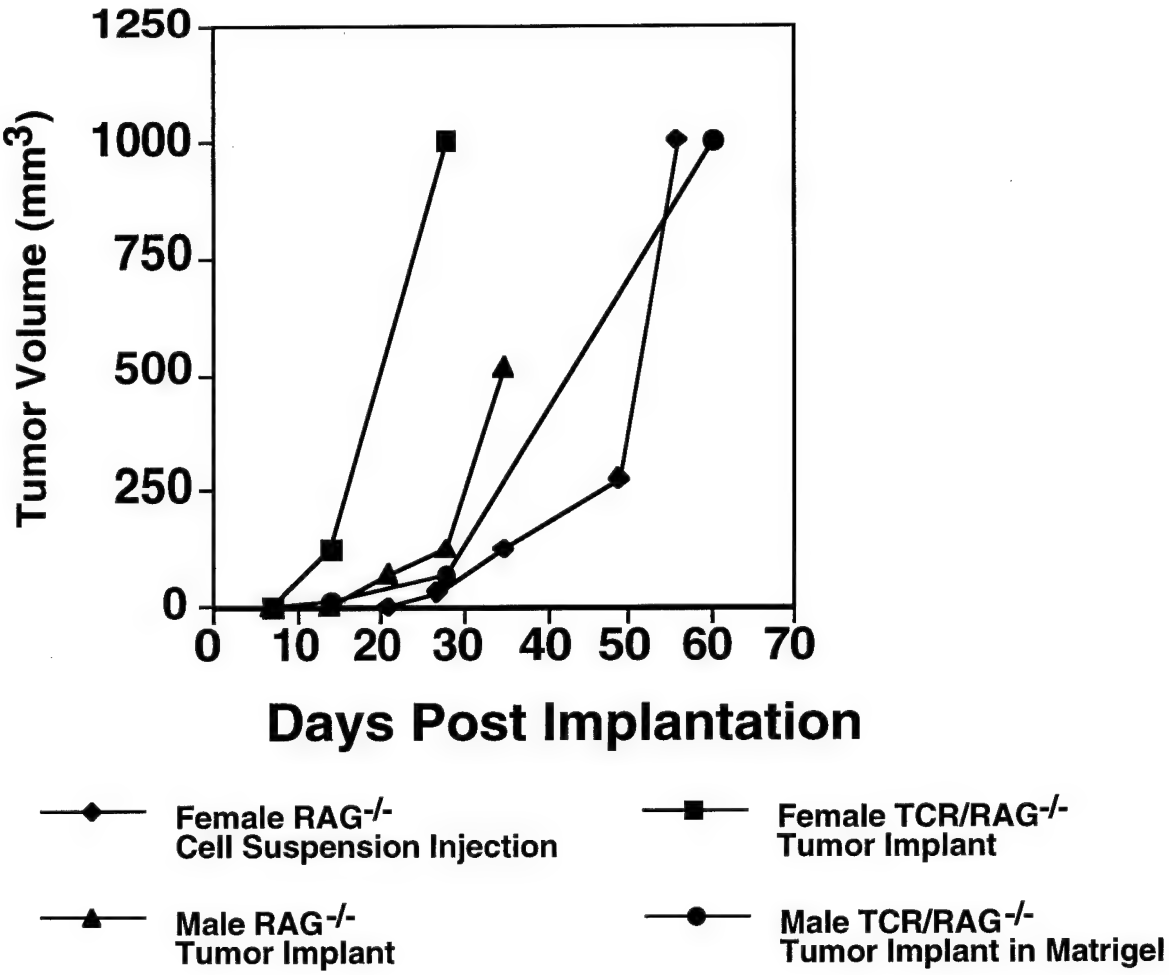
Human erbB-2^+ tumors were introduced into $TCR/RAG^{-/-}$ mice to evaluate if the transgenic animals could serve as a model for immunotherapy of erbB-2^+ cancers. SKOV-3, an erbB-2^+ ovarian carcinoma, was chosen initially because it was susceptible to CTL-mediated lysis and because several labs have used SKOV-3 in immunodeficient mice as a model for immunotherapy (13, 16). Because xenotransplantation in $RAG^{-/-}$ mice had not been reported, the SKOV-3 tumor was initially transplanted into $RAG^{-/-}$ mice. Of eleven $RAG^{-/-}$ mice injected with $2\text{-}10x10^6$ cells from tissue culture, only two had established grafts (data not shown). The reason for the inability of the tumor to take in nine of the mice was not explored. However, seven $TCR/RAG^{-/-}$ received tumor explants from the two *in vivo* tumors from $RAG^{-/-}$ mice and all established grafts (Figure 8 and data not shown). Tumors were palpable 10-20 days post injection and grew to a volume of $1000mm^3$ (1cm diameter) in approximately three weeks, at which point animals were sacrificed.

The BT-474 human breast adenocarcinoma also expresses a high level of erbB-2, was lysed by CTL 2C (Gruber and Kranz, unpublished), and has been used in SCID mice for animal models of breast cancer (26). Furthermore, BT-474 expresses estrogen receptors and has been used as a model for estrogen therapies. Thus, combination immunotherapy and hormonal therapies could be envisioned with this tumor line. To establish BT-474 grafts, $RAG^{-/-}$ and $TCR/RAG^{-/-}$ mice were implanted with estradiol pellets because of the estrogen-dependent growth of BT-474. Martigel basement membrane has been used to enhance tumor take, and was shown to be beneficial when injected with BT-474 (27). Cells were injected at a ratio of 1:1 with Matrigel basement

membrane matrix. Of the two males and two females injected, one BT-474 transplantation was successful in a TCR/RAG$^{-/-}$ female.

Together, these results demonstrate that the TCR/RAG-/- mice are capable of accepting erbB-2+ human tumor xenografts. Thus, this animal model should be useful in testing bispecific antibody agents, in the continuation of studies proposed for this project.

Figure 7. SKOV-3 and BT474 xenograft growth in TCR+/+RAG-/- mice.



CONCLUSIONS

Tasks shown below with an * have now been completed and significant progress was made on tasks indicated with an **:

***Task 1,** Cloning, expression, and testing of erbB-2 single-chain antibody, months 1-12.

***Task 2,** Screening of tumor cell lines for susceptibility to CTL-mediated lysis using the anti-fluorescein bispecific antibody, months 1-12.

***Task 3,** Breeding of transgenic TCR/RAG-2^{-} mice and testing of peripheral blood T cells for reactivity with 1B2 antibodies, months 1-20. It is anticipated that approximately 175 mice will be produced by the end of this period.

***Task 4,** Cloning, expression, and *in vitro* testing of bispecific single-chain $scFv_2$ antibody (1B2/erbB-2), months 13-24.

***Task 5,** Screening of tumor cell lines for increased susceptibility to CTL-mediated lysis when tumor cells are treated with: anti-erbB-2 antibodies, IFN-γ, TNF-α, estrogen, tamoxifen, months 13-30.

****Task 6,** Transplantation of various erbB-2^+ tumor cell lines into TCR/RAG-2^- mice and evaluation of tumor incidence, months 16-36. It is anticipated that approximately 15 mice per month will be used.

****Task 7,** Purification and *in vitro* testing of bispecific Fab_2 antibody (1B2/erbB-2), months 25-36.

Task 8, *In vivo* testing of bispecific antibodies in TCR/RAG-2- mice that have received human tumor transplants, months 30-48. It is anticipated that approximately 15 mice per month will be used.

Publications of the Principle Investigator During the Past Year (*Directly related to this project):

Schlueter, C. J., B. A. Schodin, and D. M. Kranz (1996) Specificity and Binding Properties of a Single-Chain T Cell Receptor. *J. Mol. Biol.* 256:859-869.

Brodnicki, T., P.O. Holman, and D. M. Kranz (1996) Reactivity and Epitope Mapping of Single-Chain T Cell Receptors with Monoclonal Antibodies. *Mol. Immunol.* 33:253-263.

Manning, T.C., B.A. Schodin, and D.M. Kranz (1996) A Strategy for the Synthesis and Screening of Thiol-Modified Peptide Variants Recognized by T Cells. *J. Immunol. Meth.* 192:125-132.

Schodin, B.A., T. J. Tsomides, and D. M. Kranz (1996) Correlation Between the Number of T Cell Receptors Required for T Cell Activation and TCR-Ligand Affinity. *Immunity* 5:

Patrick, T. A., D. M. Kranz, T. A. Van Dyke, and E. J. Roy (1996) Folate Receptors as Potential Therapeutic Targets in Choroid Plexus Tumors of SV40 Transgenic Mice. *J. Neuro. Oncol.* In Press.

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Schlueter, C. J., T. C. Manning, B. A. Schodin, and D. M. Kranz (1996) A Residue in the Center of Peptide QL9 Affects Binding to Both L^d and the T Cell Receptor. *J. Immunol.* In Press

*Cho, B.K., T.A. Patrick, E.J. Roy, and D. M. Kranz (1996) Efficient CTL Lysis of Folate-Receptor-Positive Tumor Cells by Single-Chain Fv/Folate Conjugates. Submitted.

*Gruber, M. M., P. G. Natali, and D. M. Kranz (1996) Lysis of Breast Cancer Cells Mediated by a Bispecific Single-Chain Anti-erbB-2 Antibody. Submitted.

*Gruber, M. M. and D. M. Kranz (1996) A Combination of γ-IFN and TNF-α Can Increase the Susceptibility of erbB-2 Positive Tumor Cells to CTL-Mediated Lysis. Submitted.

Future Work:

During the next year, we intend to complete the tasks indicated above by an ** and to begin three new tasks that we believe are important for the success of the project. These tasks will address the important questions of whether a more effective $scFv_2$ can be obtained (Tasks 9 and 10) and whether it is possible to activate CTLs in the $TCR^{+/+}RAG^{-/-}$ mice *in vivo* (Task 11).

Task 9. Characterization of the KJ16/800E6 (anti-Vβ8/anti-erbB2) bispecific $scFv_2$.

As described above, preliminary results suggest this $scFv_2$ may have higher fraction of properly folded antibodies after refolding from *E. coli* inclusion bodies, than the 1B2/800E6 $scFv_2$. This $scFv_2$ will thus be further analyzed for its ability to retarget $erbB2^+$ tumor cells *in vitro*.

Task 10. Expression of $scFv_2$ (1B2/800E6 and KJ16/800E6) in a yeast secretion system.

In collaboration with Professor Dane Wittrup at the University of Illinois, we have begun to clone the $scFv_2$ in an expression vector that has been shown to allow a high yield of active scFv to be secreted. The yeast expression system is predicted to allow only a properly folded protein to be secreted and thus we hope to be able to obtain a much higher fraction of active $scFv_2$ then in the *E. coli* system.

Task 11. Comparison of in vivo activation strategies in $TCR^{+/+}RAG^{-/-}$ mice.

Our progress in xenotransplantation of human tumors in $TCR^{+/+}RAG^{-/-}$ mice now dictates that we must decide on a method for activating T cells *in vivo* prior to bispecific antibody treatments. Three different agents will be evaluated in this effort: the anti-CD3 antibody 2C11, the superantigen Staphyloccocal enterotoxin B, and a peptide (SIYRYYGL) that has recently been shown to activate the CTL clone 2C and thus, we presume, the TCR transgenic T cells (28).

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Appendix

Three manuscripts that involve this project are provided in the Appendix:

Cho, B.K., T.A. Patrick, E.J. Roy, and D. M. Kranz (1996) Efficient CTL Lysis of Folate-Receptor-Positive Tumor Cells by Single-Chain Fv/Folate Conjugates. Submitted.

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Gruber, M. M. and D. M. Kranz (1996) A Combination of γ-IFN and TNF-α Can Increase the Susceptibility of erbB-2 Positive Tumor Cells to CTL-Mediated Lysis. Submitted.

Efficient CTL Lysis of Folate-Receptor-Positive Tumor Cells Mediated by Single-Chain Fv/Folate Conjugates[1]

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Running Title: Tumor Targeting with Antibody/Folate Conjugates

Abstract

Bispecific antibodies that bind to a tumor antigen and the T cell receptor (TCR) re-direct cytotoxic T lymphocytes (CTL) to lyse tumor cells which have escaped normal immune recognition mechanisms. One well-characterized tumor antigen, the folate receptor (FR), is expressed on most ovarian carcinomas and some types of brain cancer. Recently, we showed that conjugates of folate and anti-TCR antibodies are extremely potent bispecific agents that target tumor cells expressing the high affinity folate receptor, but not normal cells expressing only the reduced folate carrier protein. In this report, we show that the size of these conjugates can be reduced to the smallest bispecific agent yet described (30-kDa) by attaching folate to a single-chain antibody, scFv, of the anti-TCR antibody KJ16. The scFv/folate conjugates are as effective as IgG/folate conjugates in mediating lysis of FR^+ tumor cells by CTL. The optimal folate density was in the range of 5 to 15 folate molecules per scFv or IgG molecule, which yielded EC_{50} values of approximately 40 pM (1.2 ng/ml for scFv). Finally, the scFv/folate conjugates could efficiently target tumor cells even in the presence of free folic acid at concentrations that are normally found in serum. Compared to conventional bispecific antibodies, the small size of scFv/folate conjugates may prove advantageous in the ability to penetrate tumors and in reduced immunogenicity.

Introduction

It has been known for over fifty years that the immune system is capable of attacking and eliminating very large tumor burdens but sometimes fails to do so (1). Although the basis of this "escape" is incompletely understood (2), one mechanism involves the failure of tumor cells to express antigens in a context that is essential for recognition by the immune system (reviewed by Pardoll (3)). Another mechanism might be the loss of co-stimulatory ligands and adhesion molecules that aid in the recognition and activation of T cells (4).

One potential way to direct T cells or other immune effector cells against tumor cells is with bispecific antibodies (reviewed by Fanger (5)). Bispecific antibodies can be constructed to recognize two separate antigens, one on the tumor surface and the other on the surface of a cytotoxic T cell (e.g. TCR^3). Many tumor cells have potential target antigens that are tumor-specific or quantitatively more abundant on tumor cells than normal cells (tumor-associated). By bringing together the tumor cell and an activated T cell, bispecific antibodies can redirect the cytotoxicity of T cells against tumors. Previous work has demonstrated the effectiveness of bispecific antibodies against tumors *in vitro* and *in vivo* and some clinical trials have been initiated (e.g. see Refs. (6-11)). It has generally been agreed that optimizing the properties of bispecific antibodies should improve their clinical effectiveness.

Among the tumor antigens targeted with bispecific antibodies has been the high affinity folate receptor. This receptor was originally identified as a tumor-associated antigen using monoclonal antibodies that reacted with ovarian tumor cell lines (12). High affinity folate receptors (FR) are now known to be expressed at elevated levels on many human tumors, including ovarian carcinomas (e.g. one study showed that 98% of ovarian tumors express the FR, ref (13)), choroid plexus carcinomas and ependymomas (14, 15). These cancers affect a significant segment of the population: ovarian cancer is the fourth

leading cause of cancer death among women (16) and at least 30% of early childhood tumors are diagnosed as ependymomal or choroid plexus tumors (17, 18).

The high affinity FRs (K_D ~ 1 nM) differ from the lower affinity reduced folate carriers (K_D ~ 100 μM) that appear to be largely responsible for normal folate uptake as well as the transport of folate-based dihydrofolate reductase inhibitors such as methotrexate (MTX, (19-21)). The presence of FR on human tumor cells has led to the use of FR as a target for various forms of therapy with monoclonal antibodies such as MOv18 and MOv19 (12). Targeting approaches with monoclonal anti-FR antibodies have included: ^{131}I-labeled antibodies (22), engineering of constant regions to optimize antibody-dependent cellular cytotoxicity (23), and bispecific antibodies that target immune effector cells to the FR^+ tumor (24-26). The latter studies have used anti-FR antibodies linked to either anti-Fc receptor antibodies or to anti-CD3 antibodies for recruitment of monocyte/natural killer cells or cytotoxic T cells, respectively. Clinical trials with the radiolabeled antibodies and the anti-FR/anti-CD3 bispecific antibodies have recently been initiated (9, 11, 22). Delivery of toxins and antisense nucleotides to the interior of malignant cells using the endocytotic properties of the FR have also been undertaken (27, 28).

Monoclonal antibodies to the mouse FR have not been produced and thus antibody targeting of the FR on mouse tumor cells as a model for human disease has not been possible. Nevertheless, two mouse homologs of the human FR isoforms have been identified and these receptors also bind folate with high affinity (K_D ~ 1 nM) (29, 30). Several mouse leukemia lines have been selected for high FR expression by growth in media with low concentrations of folate. Two forms (α and β) of the mouse FR have been identified as 38-kDa lipid-linked membrane glycoproteins (31). As in humans, FR also appear to be expressed at high levels on some mouse tumors. For example, we have recently found that mouse choroid plexus tumors that arise in SV40 transgenic mice (32) express high levels of FR (33).

The high affinity of folate for FR suggested to us that attachment of folate directly to an anti-TCR antibody might efficiently target FR^+ tumor cells for lysis by T cells. We recently reported that such conjugates have very potent targeting activity without adversely affecting normal cells that express only the reduced folate carrier protein (34). However, it is reasonable to predict that the most effective agents for targeting solid tumors will have reduced sizes that allow greater tumor penetration. For instance, comparative bio-distribution studies with ^{125}I-labeled IgG, $F(ab)_2$, Fab fragments and scFv in human colon carcinoma xenografts in athymic mice demonstrated that scFv penetrated tumor faster, deeper, and more uniformly than other forms of the antibody (35).

In this report, a 29-kDa scFv of the anti-Vβ8 antibody KJ16 (36) was conjugated with folate, and its targeting potential was evaluated *in vitro*. Cytotoxicity assays with these preparations showed that lysis of mouse FR^+ tumor cells was highly specific and correlated directly with FR density ($r = 0.93$). Comparison between folate labeled-IgG and scFv demonstrated that both conjugates have nearly identical targeting efficiencies (EC_{50} = 40 pM) and lysis with scFv/folate could be detected at concentrations as low as 1 pM. Direct competition experiments with free folate demonstrated that the scFv/folate conjugate could effectively target FR^+ tumor cells even at folate concentrations above normal serum levels. The reduced size of the scFv/folate compared to other bispecific reagents as well as it high potency suggests that it has potential for *in vivo* therapy. In addition, the conjugate may serve as a model for the development of future novel bispecific agents that contain small ligands specific for tumor cell surface antigens.

Materials and Methods

Cell Lines and Antibodies. The following DBA/2-derived tumor cell lines were maintained in RPMI 1640 containing 5 mM HEPES, 10% fetal calf serum, 1.3 mM L-glutamine, 100 units of penicillin per ml, 100 μg per ml streptomycin, and 50 μM 2-mercaptoethanol: Mel, murine erythroleukemia cell (37); La, a subline of Mel selected on

low folate (31); L1210, a murine leukemia cell line (38); and F2-MTX^{r}A, a MTX resistant subline of L1210 selected for increased expression of FR-β by growth on low folic acid (39). La expresses primarily the α isoform of folate receptor (FR), F2-MTX^{r}A expresses only the FR-β isoform, and L1210 expresses both α and β isoforms. CTL clone 2C, a Vβ8$^+$ alloreactive cell line specific for L^d (40), was maintained in the same RPMI media supplemented with 10% (vol/vol) supernatant from concanavalin A-stimulated rat spleen cells, 5% α-methyl mannoside, and mitomycin-C treated BALB/c mouse spleen cells as stimulators. KJ16 is a rat IgG antibody specific for the Vβ8.1-2 domains of the TCR (41) and was provided by Drs. Kappler and Marrack. KJ16 monoclonal antibody was prepared from tissue culture supernatant generated in a Vita-Fiber mini flow path bioreactor (Amicon) and concentrated by precipitating twice in 50% ammonium sulfate. KJ16 Fab fragments, FITC-labeled Fab fragments, and KJ16 scFv were generated and purified as described previously (36). Briefly, scFv was refolded from inclusion bodies and monomeric scFv was purified by G-200 HPLC purification. Monoclonal antibody 30.5.7 is specific for the major histocompatibility complex (MHC) class I product L^d (42) and was prepared as ascites fluid and used without further purification in some cytotoxicity assays.

Preparation of Folate/Antibody Conjugates. Folate was coupled through its carboxyl groups to antibody amine groups using a carbodiimide procedure described previously (34, 43). Unless indicated, a 5-fold molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Pierce) was added to folate (Sigma) dissolved in dimethyl sulfoxide. After 30 minutes at room temperature in the dark, a 20- to 700- fold molar excess of the EDC-activated folate was added to 0.1 - 0.5 mg of antibody in 0.1 M MOPS, pH 7.5. After 1 hour at room temperature, the sample was either applied to a Sephadex G-25 column pre-equilibrated in 0.1 M MOPS or immediately dialyzed into phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.3). If passed over a G-25 column, the excluded-peak fractions were pooled and dialyzed against PBS. After dialysis, protein concentrations were determined using the

bicinchoninic assay (Pierce Chemical Co.) using a protein A purified intact mouse antibody as the standard. Antibody conjugates were also analyzed spectrophotometrically at 363 nm and the density of the folate per antibody was calculated by dividing the molar concentration of folate on the conjugate ($A363/\varepsilon_M$; $\varepsilon_M = 6197\ M^{-1}$) by the antibody concentration. Folate densities obtained ranged from ~1 folate/antibody to ~120 folates/antibody. Conjugates were stored at 4°C in the dark.

Mass Spectrometry. Mass spectra were obtained on a TofSpec using electrospray ionization. Samples were dialyzed against 1 mM potassium phosphate buffer, pH 8.0 and concentrated to 10 to 25 pmol/ml. Analysis was performed by the Mass Spectrometry Laboratory, School of Chemical Science, University of Illinois.

Folate Binding Assays. Binding assays were conducted by using ^{125}I-labeled folate (NEN; specific activity = 2200 Ci/mmol; 1 Ci = 37 GBq). Cells were washed with PBS containing 0.1% bovine serum albumin, pH 7.3 (PBS-BSA), to remove excess free folate present in the cell culture medium. Cells, labeled folate, and competitors were incubated in triplicate in 50µl of PBS-BSA for 1 hour at 37°C. Incubation at 37°C has previously been shown to produce levels of binding similar to that obtained with acid pre-treatment (34). Samples were loaded into tubes containing 300µl oil [80% (vol/vol) dibutyl phthalate/20% (vol/vol)olive oil] and bound and free ligand were separated by a 3 second centrifugation at 12,000 x *g*. Tubes were frozen and cut to allow the radioactivity in the cell pellet and supernatants to be quantitated separately.

T Cell Receptor Binding Assays. The relative affinity of the scFv/folate conjugates for the TCR was determined by a competition assay with 5-aminofluorescein isothiocyanate (FITC) labeled KJ16 Fab fragments as previously described (36). In brief, various concentrations of antibody were added to triplicate sets of $6x10^5$ 2C cells in the presence of a constant amount of FITC-labeled Fab fragments. After a 30 minute incubation on ice, the entire mixture (antibody + FITC-labeled Fab fragments + 2C cells) was passed through a flow cytometer without washing. Inhibition by various KJ16 preparations was measured

by quantitating the decrease in bound fluorescence by flow cytometry (performed with a Coulter Electronics EPICS 752 at the University of Illinois Biotechnology Center). The concentrations of unlabeled antibody giving 50% inhibition (IC_{50}) were determined relative to the maximum fluorescence (in the absence of inhibitor) and the background fluorescence (in the presence of a large excess of intact antibody).

Cytotoxicity Assays. Tumor cells were labeled with 50-100 µl of ^{51}Cr (2.5 mCi/ml) for 60 minutes at 37°C, washed twice with folate-free RPMI 1640 medium containing 5% (vol/vol) fetal calf serum (folate-free media), and used in 96-well plate cytotoxicity assays at 10^4 cells per well. Because each of these cell lines also expressed the alloantigen L^d which is recognized by CTL 2C, assays were performed in the presence of anti-L^d antibody to minimize non-FR-mediated lysis. Ascites of anti-L^d antibody 30.5.7 was diluted 1:100 into folate-free media containing effector cells (2C). Effector cells were added to target cells at an effector-to-target cell ratio of 5:1. Antibodies and folate/antibody conjugates were diluted in folate-free media and added to triplicate wells at various concentrations. Plates were incubated at 37°C for 4 hours in 5% CO_2, and supernatants were removed for γ counting. For the inhibition of scFv/folate by free folate, a nonsaturating concentration of scFv/folate (3 nM) that would generate maximal cytotoxicity was used together with various concentrations of free folate. Unless indicated otherwise, the specific release mediated by the folate conjugates was determined by subtracting the release in the absence of the conjugates (e.g. % specific release = {experimental counts- spontaneous counts}/{maximal counts - spontaneous counts} x 100).

Results

Characterization of scFv/Folate Conjugates. The scFv of KJ16, an anti-Vβ8 antibody, was purified from *E. coli* inclusion bodies after guanidine denaturation, refolding, and HPLC gel filtration. Purified scFv migrated as an apparent 35-kDa protein on SDS-PAGE gels (Figure 1A). Folate was coupled to the scFv using the carbodiimide

(EDC) reaction which links carboxyl groups of folate to primary amine groups on the protein. In engineering the scFv, the V_L and V_H domains were joined by the 26 residue linker, 205s, that contains 8 lysine residues (36, 44). We reasoned that the presence of multiple lysine residues in a highly accessible, solvent exposed region may result in higher folate densities in the linker region as opposed to the antibody V regions. In initial studies, folate was coupled to the scFv at a 100:1 folate to antibody molar ratio. Under these conditions, scFv/folate preparations contained an average of 3 to 8 folates per antibody (N = 3), based on spectrophotometric analysis. Consistent with this finding, migration of the scFv/folate conjugate on SDS-PAGE gels was slightly slower than scFv and the band was more diffuse (Figure 1A).

The folate density determined by photometry does not provide information about the heterogeneity of the conjugates. To determine the range of epitope densities within a single preparation, scFv/folate conjugates were examined by electrospray ionization mass spectrometry (Figure 1B). Unlabeled scFv demonstrated a single peak with a molecular mass of 29,082-Da. In contrast, folate-labeled scFv existed as a collection of antibody populations, each differing by the molecular weight of a folate molecule (~400-Da), detectable up to 7 folates per antibody. Integration of mass spectra showed that greater than 85% of the scFv molecules were labeled with one or more folate molecules. Folate densities estimated from mass spectra were generally 1.5 to 2-fold lower than densities estimated by spectrophotometry. This could in part be due to lower solubility of high density conjugates under conditions required for mass spectrometry or to the dissociation of some folate molecules during ionization.

Binding of scFv/Folate Conjugates to the T Cell Receptor. To examine if folate conjugation affected the binding of scFv antibodies to the TCR, a scFv/folate conjugate was compared with unlabeled scFv. In a competitive flow cytometric assay, fluorescein labeled KJ16 Fab fragments were inhibited from binding the Vβ8-positive CTL clone 2C by unlabeled Fab fragments, scFv, or scFv/folate (Figure 2). As shown previously, scFv

antibodies have an approximate 3-fold higher apparent affinity than Fab fragments, possibly because of the presence of noncovalently associated scFv dimers (36). Comparison of folate-labeled and unlabeled scFv showed that the folate conjugate had an apparent affinity ~3-fold lower than scFv (i.e. approximately equal to KJ16 Fab fragments). This decreased binding, compared to unlabeled scFv, could be due either to chemical modification of active site residues with folate or to the interference of dimer formation by folate. The fact that folate conjugates bound the TCR as well as KJ16 Fab fragments, which have a K_D of ~130 nM (36, 45), indicated that the conjugates have potential to mediate lysis of target cells by CTLs.

Binding of scFv/Folate Conjugates to Folate Receptors on Tumor Cells. The ability of scFv/folate conjugates to bind folate receptors (FR) on the surface of tumor cells was examined by a competition binding assay using ^{125}I-labeled folate as the labeled ligand (Figure 3). The competition assay used the F2-MTX^{r}A cell line that expresses the β isoform of the FR. Competitors included various concentrations of free folate, unlabeled scFv, and three different scFv/folate preparations. Examination of the binding curves showed that folate conjugated antibody, but not unlabeled scFv, binds to the FR$^+$ tumor cell line. However, based on molar folate concentration, the folate conjugates had a relative affinity that was approximately 10 to 30 fold less than free folate. This decrease in apparent affinity was consistent with previous observations (34) and could be partly attributed to the carbodiimide labeling procedure. This procedure links folate through either the α or γ carboxyl groups but only linkage through the γ-carboxyl retains binding (46). It is also likely that some conjugated folates might be sterically hindered from interacting with a FR, either because they are too close to a neighboring folate or because of the location of nearby amino acid residues that prevent binding.

CTL-Mediated Lysis of FR$^+$ Tumor Cells by scFv/Folate Conjugates. The specificity and efficiency of tumor targeting with scFv/folate conjugates were examined in a ^{51}Cr release assay with CTL clone 2C (Figure 4). Four different tumor cell lines that have

a range of cell surface FR densities were used as target cells: F2-MTX^{r}A (200,000 sites/cell); La (60,000 sites/cell); L1210 (8,000 sites/cell); Mel (no-detectable FR). Each of the FR$^+$ cell lines was lysed in the presence of the scFv/folate conjugate and the extent of lysis was directly correlated with the expression level of the FR ($r = 0.93$). Lysis was completely inhibited by free folate indicating that targeting of the tumor cells was specifically mediated by the folate receptor and not some other cell surface molecule. The lysis mediated by these conjugates was highly specific (e.g. the FR$^-$ cell line Mel was not lysed by the conjugate even at concentrations over 10^5-times that required for detectable killing of the FR$^+$ cell line F2-MTX^{r}A) and extremely potent (e.g. lysis was detectable at concentrations as low as 1 pM of scFv/folate). Thus, the presence of reduced folate carrier protein (as present in Mel, and in all other normal cells) does not result in cell destruction. It is important to point out that the FR density reportedly on ovarian tumors is even higher (~1 million/cell) than those on these tumor cell lines (47).

Effects of Folate Density on Targeting. To examine the effects of folate density and labeling on the targeting efficiency of scFv/folate conjugates, the antibody was labeled with folate under various carbodiimide-mediated coupling conditions. The carbodiimide EDC couples folate through the free carboxyl groups, but when used in the presence of protein it may also lead to protein modification and subsequent precipitation or inactivation. To evaluate the optimal levels of EDC for folate coupling, several different concentrations of EDC were used at a constant concentration of folate during coupling. Purified conjugates of scFv/folate prepared at three different EDC concentrations were examined in the cytotoxicity assay to determine the concentrations that yielded maximal specific targeting (Figure 5A). EDC used at the two lowest levels yielded conjugates with approximately equal targeting efficiency but higher levels yielded conjugates with reduced efficiency and frequently led to protein precipitation (Figure 5A and data not shown).

To directly examine the effect of folate density, activated folate was prepared at a constant EDC:folate ratio (5:1) and conjugates were produced by adding different ratios of

activated folate to the scFv protein. After dialysis to remove unreacted folate and excess EDC, conjugates were evaluated by spectrophotometry to determine folate densities and cytotoxicity assays to evaluate targeting efficiency. Folate densities ranged from approximately 1 to 20 folates per scFv. As shown in Figure 5B, each of the conjugates was capable of mediating lysis of the FR^+ tumor cell line by CTL clone 2C. However, conjugates with either the lowest density (1.3 folates/scFv) or highest density (20.4 folates/scFv) were 5 to 10-fold less effective than conjugates with intermediate folate densities. This experiment has been performed several times with similar results, indicating that the optimal folate density appears to be in the range of 5 to 15 folates per scFv, as determined by spectrophotometry. The reduction in targeting efficiency at higher folate densities is not due to the inability of these conjugates to bind to FR^+ cells (Figure 3), but is likely a consequence of chemical modification of amino acid residues important in scFv binding or stability.

Comparison of Intact Antibody and scFv. To determine the relative effectiveness of intact KJ16 IgG versus scFv-KJ16, both forms were labeled with folate at a 100:1 molar ratio of activated folate to antibody and under identical EDC reaction conditions (33 mM EDC, 6.7 mM folate). These conditions yielded folate densities of 7 and 5 for the scFv and intact antibody, respectively. Cytotoxicity assays with these preparations showed nearly identical targeting efficiencies for the intact and scFv forms of KJ16 (Figure 6). The concentration required to obtain 50% of the maximal specific release (EC_{50}) was approximately 40 pM (1.2 ng/ml for scFv). Comparison of intact and scFv conjugates in an ^{125}I-folate binding assay indicated no significant difference in their ability to bind FR (data not shown).

Intact KJ16 antibody was also labeled at various folate densities to determine if targeting efficiency could be optimized further. Conjugates were again evaluated by spectrophotometry to determine folate densities and cytotoxicity assays to evaluate targeting efficiency (Figure 7). Folate densities ranged from approximately 4 to 126 folates per

antibody molecule. As shown with scFv preparations, high and low folate density resulted in decreased targeting effectiveness for the intact antibody. As with scFv preparations, the optimal densities appear to be in the range of 5 to 15 folates per antibody, as determined by spectrophotometry.

Inhibition of scFv/Folate Conjugate-Mediated Lysis by Free Folate. Normal serum folate may reduce the effectiveness of the scFv/folate conjugate by competing for the folate receptor *in vivo*. A ^{51}Cr release assay was used to evaluate the effectiveness of the scFv/folate conjugate-mediate lysis at biologically relevant concentrations of free folate (Figure 8). Free folate was diluted in folate-free media and various concentrations were added to triplicate wells containing ^{51}Cr labeled F2-MTX^{r}A cells, 2C effector cells, and a fixed, nonsaturating concentration of scFv/folate conjugate (3 nM) that would generate maximal specific release. The resulting titration curve demonstrated that at normal human serum folate levels (9-36 nM, (48, 49)) the scFv/folate conjugate retained most of its activity. For instance, at 20nM folate, the scFv/folate conjugate exhibited over 80% of its CTL-mediated targeting potential. Although murine serum folate levels are significantly higher than normal human serum values (124 to 700 nM) due to folate-rich chow (50, 51)), even at these elevated folate concentrations, the scFv/folate conjugate exhibited 30 to 60% of its targeting potential.

Discussion

The most effective agents for targeting solid tumors will likely have reduced sizes that allow greater tumor penetration. This report characterizes the smallest bispecific agent yet described for redirecting the activity of immune effector cells against tumors. Initial bispecific antibody studies to target ovarian tumors that express high affinity folate receptors have used intact heterobifunctional antibodies (~150 kDa) that bind to CD3 and the FR. These agents showed efficacy in animal models and they have recently entered testing in clinical trials (9, 11, 22). Although not yet reported for anti-FR antibodies,

several labs have shown that it is possible to engineer smaller bispecific antibodies of ~60 kDa by linking two scFv regions (52-54). Here we show that the size of a bispecific targeting agent can be reduced even further to ~30 kDa for the scFv/folate conjugates. Furthermore, the targeting efficiency of the engineered scFv/folate conjugate is comparable to that of the native intact antibody/folate conjugates.

For coupling of folate to the anti-Vβ8 scFv KJ16, a carbodiimide reaction that links the carboxyl groups of folate to the amino groups of the antibody was used. KJ16 scFv binds to the cell surface TCR with an affinity that is similar to the intact KJ16 antibody (36). The V_L and V_H regions of the scFv are linked by a 26 amino acid region that contains 8 lysine residues. We reasoned that this charged linker would be accessible to folate, would be located distal to the binding site, and would contain multiple attachment sites for folate through the EDC reaction. Under coupling conditions where folate concentrations are nonsaturating, the scFv and intact antibody have comparable densities of folate attached per molecule (~5 to 15 folates per molecule), despite the 5-fold greater size of the intact antibody. Cytolytic assays demonstrated that these conjugates have very similar targeting efficiencies (Figure 6).

Although folate is probably attached to lysines present in the linker, the scFv/folate conjugates are actually heterogeneous populations as evidenced by mass spectra (Figure 1B). Within a scFv/folate preparation there are most likely conjugates with enhanced targeting properties and conjugates with diminished targeting properties. The former could include not only those with folate at an accessible location, but perhaps those with multiple folates that allow for multivalent interactions with FRs on tumor cells. Conjugates which have diminished bispecific properties would include those that have folate attached through amine groups in the scFv active site or in regions which destabalize the V_L-V_H interaction. These are analogous to those preparations derived with very high folate densities, where the targeting efficiency is significantly reduced (Figures 5B and 7).

Several considerations suggest that further optimization of the scFv/folate conjugates could yield even more potent agents. The anti-TCR antibody KJ16 has a relatively modest K_D of ~100 nM (36). Our lab is currently engineering higher affinity scFv antibodies by phage display; antibodies with $K_D \leq 1$ nM have been routinely generated using this approach (55). In addition, the affinity of the scFv/folate conjugate for the FR^+ tumor cells was up to 30 fold less than the affinity of free folate for the FR (Figure 3). Coupling of folate through the γ-carboxyl, plus homogeneous linkage perhaps through cysteine or multiple cysteines, should improve the affinity for the FR. Alternatively, other folate analogs with higher affinity than folate could be employed (56). The use of these strategies should allow the development of scFv conjugates that have EC_{50} values considerably less than those described in this report.

It is of significant note that the scFv agent described here is not only the smallest agent but is at least as potent *in vitro* as bispecific antibodies described in the literature. For example, the EC_{50} of other bispecific agents range from 1 ng/ml to 100 ng/ml (47, 57-59). Various other antibody or folate-based targeting agents have EC_{50} values that range from 0.1 ng/ml to 200 ng/ml (e.g. Refs. (43, 46, 60-63)). *In vitro* assays for these other agents typically involved 24 hour incubation periods while the cytotoxicity assays for CTL-mediated lysis, described here and elsewhere, are four hour incubations. Thus, there is reason to believe that the scFv/folate conjugate has considerable promise *in vivo*: It has an EC_{50} of approximately 1 ng/ml, is smaller than the other agents, and remains effective at folate levels found in normal human serum.

The fact that the scFv/folate conjugate is in direct competition with serum folate brings about the intriguing possibility of modulating the effectiveness of scFv/folate conjugate treatment by altering the levels of serum folate. For instance, recent studies with mice have shown that serum folate can be intentially decreased up to 100-fold with special low-folate diets (50, 51). The decreased folate concentration greatly enhanced the ability of a ^{67}Ga-labeled deferoxamine-folate conjugate to image FR^+ tumors *in vivo* (50). We

envision that similar low-folate diets will likewise enhance the therapeutic effectivness of the scFv/folate conjugate. Conversely, serum folate could be increased in situations where non-specific T cell interactions lead to adverse side-effects. Thus, the use of folate as the small molecule ligand specific for a tumor antigen may allow for additional levels of regulation normally not available with other immunotargeting agents.

In vivo testing is currently underway in SV40 transgenic mice that develop choroid plexus tumors exhibiting elevated levels of the high affinity folate receptor (33). Preliminary results indicate that T cells specifically infiltrate the tumor after treatment with the scFv/folate conjugate (B.K.C., T.A.P., D.M.K., and E.J.R. unpublished data). At this time, it is unclear whether conjugate-bound-T cells extravasate into the tumor or if T cells first extravasate into the tumor and subsequently recognize the conjugate bound to cell surface FR. The latter mechanism would favor the enhanced tumor-penetration characteristics of the smaller scFv molecule and would likely lead to increased therapeutic effectiveness.

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[3] The abbreviations used are: CTL, cytotoxic T lymphocyte; FR, folate receptor; TCR, T cell receptor; scFv, single-chain antibody binding domain; MHC, major histocompatibility complex; K_D, dissociation constant; SV40, simian virus 40; EC_{50}, concentration of antibody-folate conjugate required for half-maximal specific release; fol, folate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; FITC, 5-aminofluorescein isothiocyanate; Ga, gallium; Fab, antigen binding fragment derived from papain digestion of Ig molecule; Fc, effector region of an antibody derived from papain digestion of Ig molecule; V regions, variable regions of IgG heavy and light chains; ε, extinction coeffecient; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, 10 mM phosphate buffer, 150 mM NaCl, pH 7.3; PBS-BSA, PBS containing 0.1% bovine serum albumin; MTX, methotrexate.

Legends to Figures

Figure 1. **SDS-PAGE analysis and mass spectra of purified preparations of scFv KJ16 and scFv/folate conjugate.** *A*, Samples were electrophoresed through a 10% polyacrylamide gel under reducing and non-reducing conditions and proteins were visualized by staining with Coomassie Blue. *B*, Samples were concentrated, dialyzed against 1 mM potassium phosphate buffer, pH 8.0, and mass spectra were obtained on a TofSpec using electrospray ionization. Purified scFv existed as a single species with molecular mass 29,082-Da. In contrast, folate-labeled scFv existed as a collection of antibody populations, each differing by the molecular weight of a folate molecule (~400-Da), detectable up to 7 folates per antibody.

Figure 2. **Binding of KJ16 scFv/folate to cell surface T cell receptor.** The binding of FITC-labeled KJ16 Fab fragments to the Vβ8-positive T cell clone 2C was inhibited by purified scFv/folate, scFv, or Fab fragments. $6x10^5$ 2C cells were incubated 30 min at 4°C with FITC-labeled KJ16 Fab fragments and various concentrations of folate-labeled scFv KJ16 (▲), unlabeled scFv (■), and unlabeled Fab fragments (●). A relative affinity of the scFv/folate was determined by comparing the concentrations required to inhibit 50% of the FITC-labeled Fab fragments from binding the 2C TCR.

Figure 3. **Binding of KJ16 scFv/folate to folate receptors.** ^{125}I-labeled folate was incubated with F2-MTX^{r}A cells in the presence or absence of competitors for 1 h at 37°C. Concentrations refer to folate rather than antibody concentrations. Competitors included free folate (□) and KJ16 scFv/folate conjugates with different folate densities; 2.8 fol/scFv (O), 9.2 fol/scFv (■), and 20.4 fol/scFv (▲). Inhibition was not observed in the absence of competitor (Δ) or in the presence of unconjugated scFv (●) (error shown is ± average SEM).

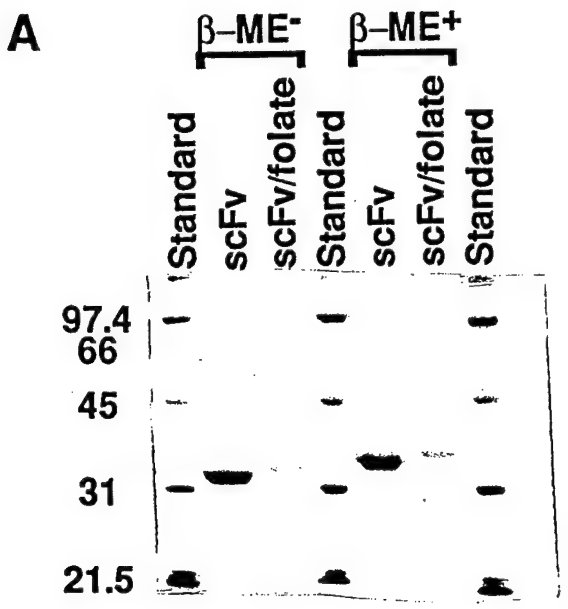
Figure 4. **Cytoxicity assay of various tumor cells lines with the scFv/folate conjugate and CTL clone 2C.** Various concentrations of the scFv/folate conjugate were incubated with ^{51}Cr labeled tumor cells and CTL 2C for 4 h at an effector-to target ration of 5:1. Experiments were performed in the presence of anti-L^d antibody to minimize lysis due to recognition of L^d, the nominal ligand for CTL 2C. Lysis correlated directly with the level of FR expressed by the cell line: F2-MTX^{r}A (200,000 sites/cell, ▲); La (60,000 sites/cell, ■); L1210 (8,000 sites/cell, ●); Mel (no-detectable FR, O). Assays with free folate at a final concentration of 1.5 μM were performed with the scFv/folate conjugate at a concentration of 0.09 nM (+ Folate).

Figure 5. **Effect of folate density on targeting with scFv/folate conjugates.** *A*, To determine the EDC concentration that yielded the optimal targeting efficiency, three different EDC concentrations were evaluated ([EDC] = 13 mM, ● ; [EDC] = 65 mM, ■ ; [EDC] = 260 mM, ▲). Conjugates of scFv/folate were prepared at the indicated concentrations of EDC, at constant folate concentration, and used in cytotoxicity assays with the tumor cell line F2-MTX^{r}A and CTL 2C. *B*, scFv/folate conjugates with different folate densities were prepared by labeling scFv protein with various amounts activated folate, using an EDC:folate ratio of 5:1 (EDC =65 mM). These conjugates were tested in cytoxicity assays using F2-MTX^{r}A and CTL 2C. Folate densities were: 1.3 fol/scFv, ● ; 4.0 fol/scFv, ■ ; 9.2 fol/scFv, ▲ ; 9.3 fol/scFv, O ; 20.4 fol/scFv, Δ .

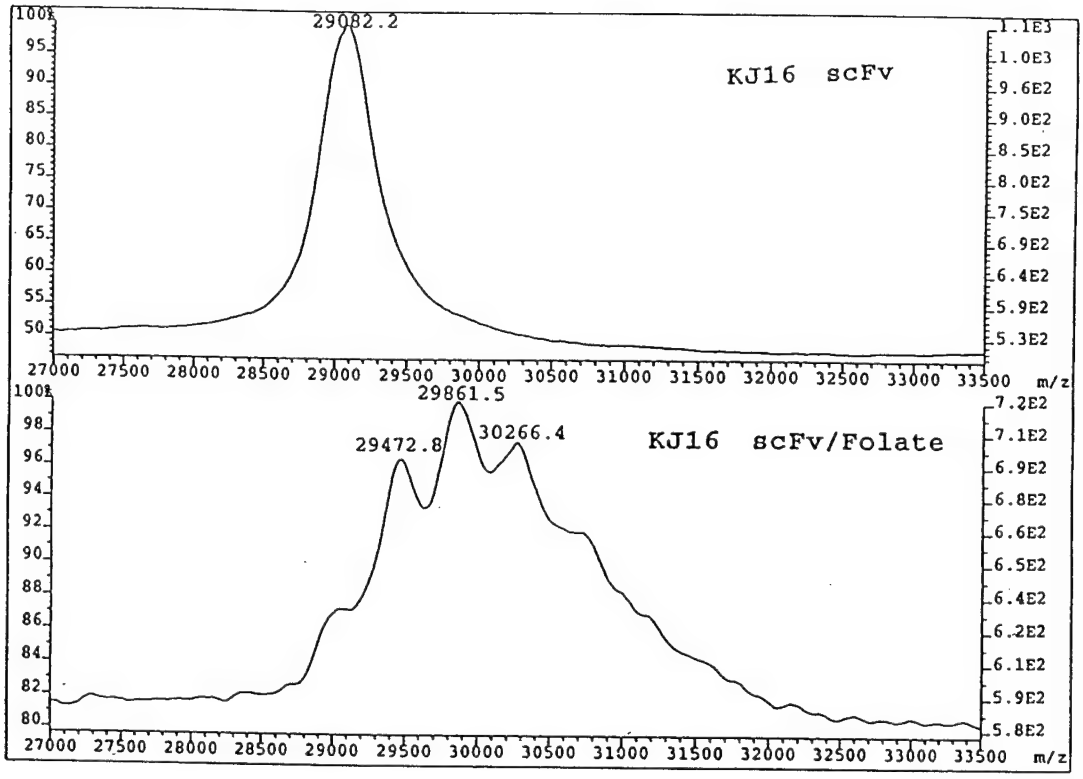
Figure 6. **Comparison of scFv/folate and IgG/folate in CTL-mediated lysis of tumor cells.** Folate was conjugated to KJ16 scFv and intact antibody under identical conditions (3.3 mM EDC, 100:1 molar ratio of folate:antibody) yielding conjugates with 7 fol/scFv (●) and 5 fol/IgG (■). Cytotoxicity assays were performed with these conjugates using ^{51}Cr-labeled F2-MTX^{r}A cells and CTL clone 2C.

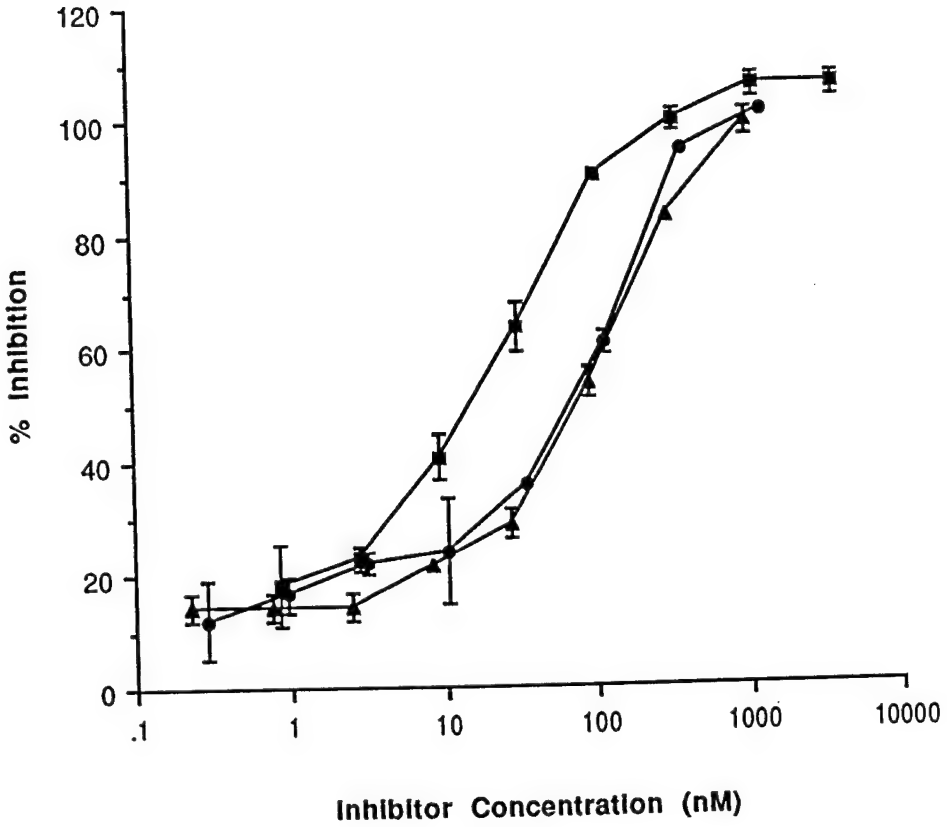
Figure 7. **Effect of folate density on targeting with IgG/folate conjugates.** Intact antibody was labeled at several folate concentrations to determine if, like scFv, a specific range folate density would yield the optimal targeting effectiveness. Conjugates with the following densities: 4.5 fol/IgG, □ ; 7.0 fol/IgG, ● ; 13.6 fol/IgG, ▲ ; 56.7 fol/IgG, ■ ; and 126.2 fol/IgG, O were assayed with ^{51}Cr-labeled F2-MTX^{r}A cells and CTL clone 2C.

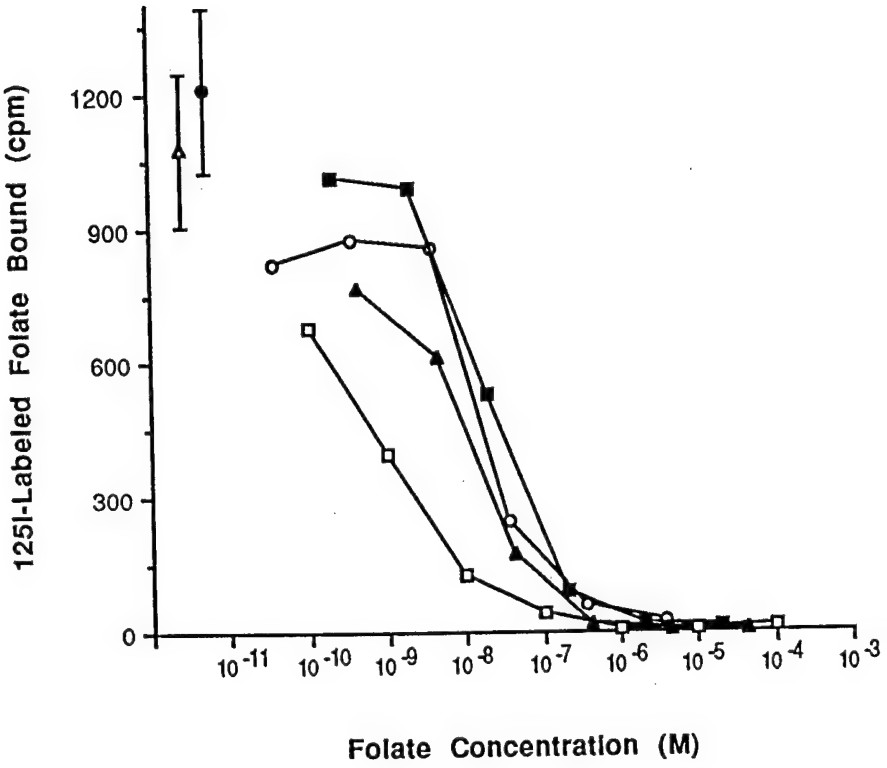
Figure 8. **Inhibition of scFv/folate-mediated lysis by free folate.** Various concentrations of free folate were added to triplicate wells containing ^{51}Cr labeled F2-MTX^{r}A cells, 2C effector cells, and a fixed, nonsaturating concentration of scFv/folate conjugate (folate density = 8) that would yield maximal specific release (3 nM). Inhibition was calculated as a function of the specific release in the absence of folate competitor. Normal human serum folate concentrations range from 9-36 nM (48, 49).

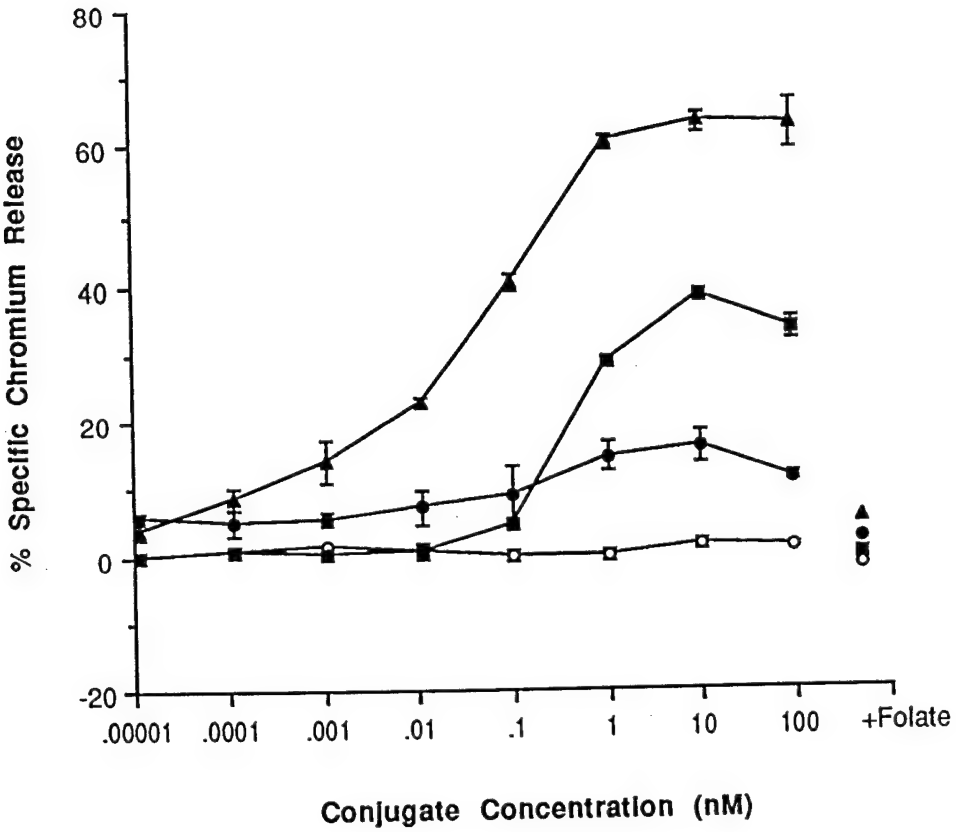

A
β-ME-
β-ME+
Standard
scFv
scFv/folate
Standard
scFv
scFv/folate
Standard
97.4
66
45
31
21.5

B

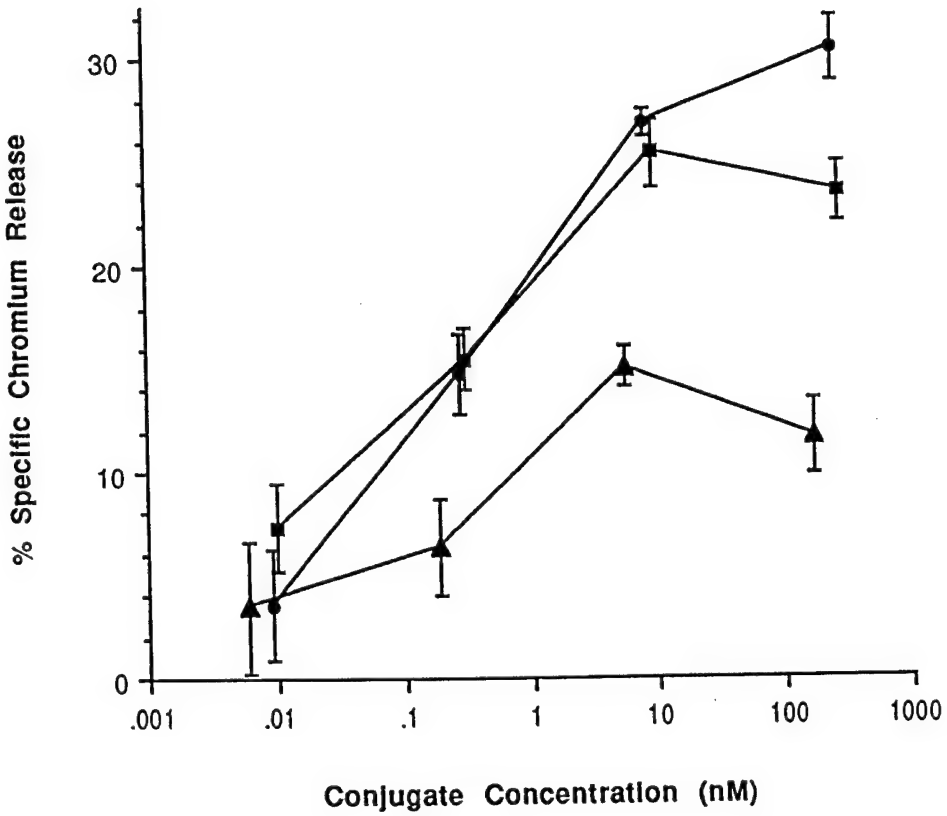



120
100
80
60
40
20
0
% Inhibition
.1
1
10
100
1000
10000
Inhibitor Concentration (nM)


125I-Labeled Folate Bound (cpm)
1200
900
600
300
0
10-11
10-10
10-9
10-8
10-7
10-6
10-5
10-4
10-3
Folate Concentration (M)


80
60
40
20
0
-20
% Specific Chromium Release
.00001
.0001
.001
.01
.1
1
10
100
+Folate
Conjugate Concentration (nM)

A


% Specific Chromium Release
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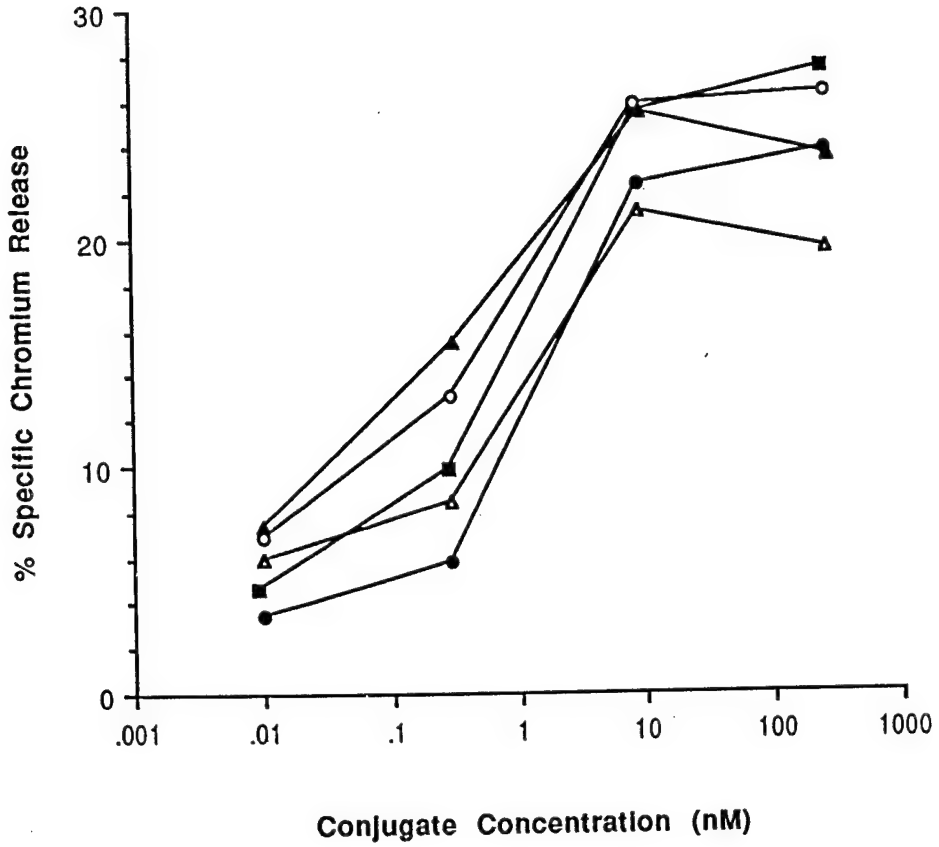
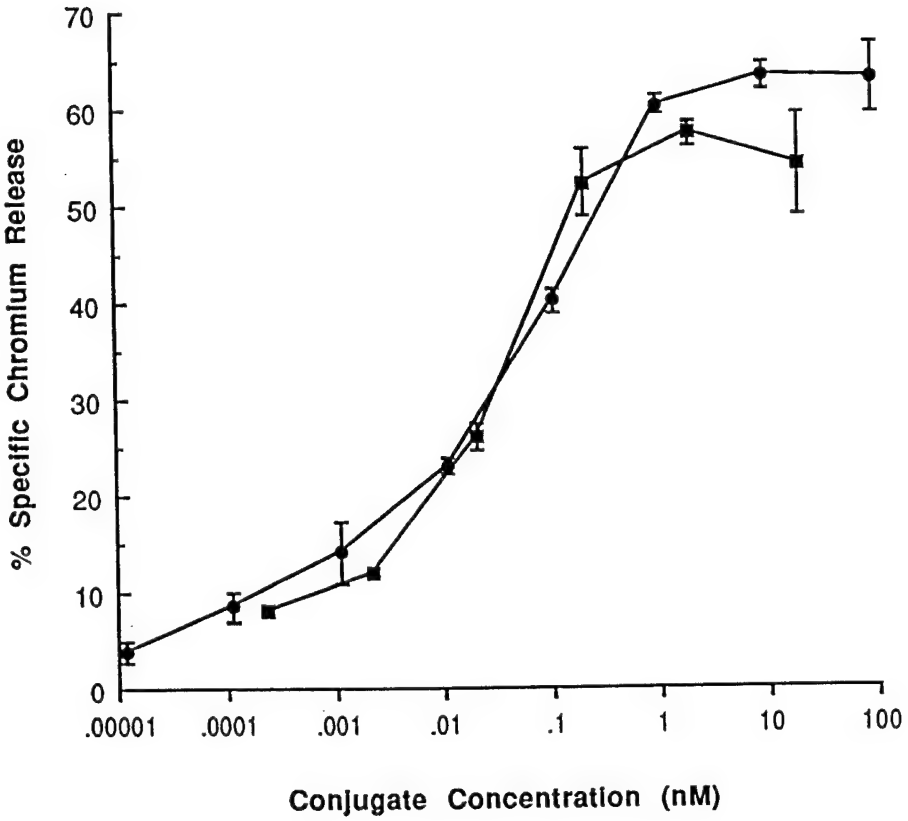
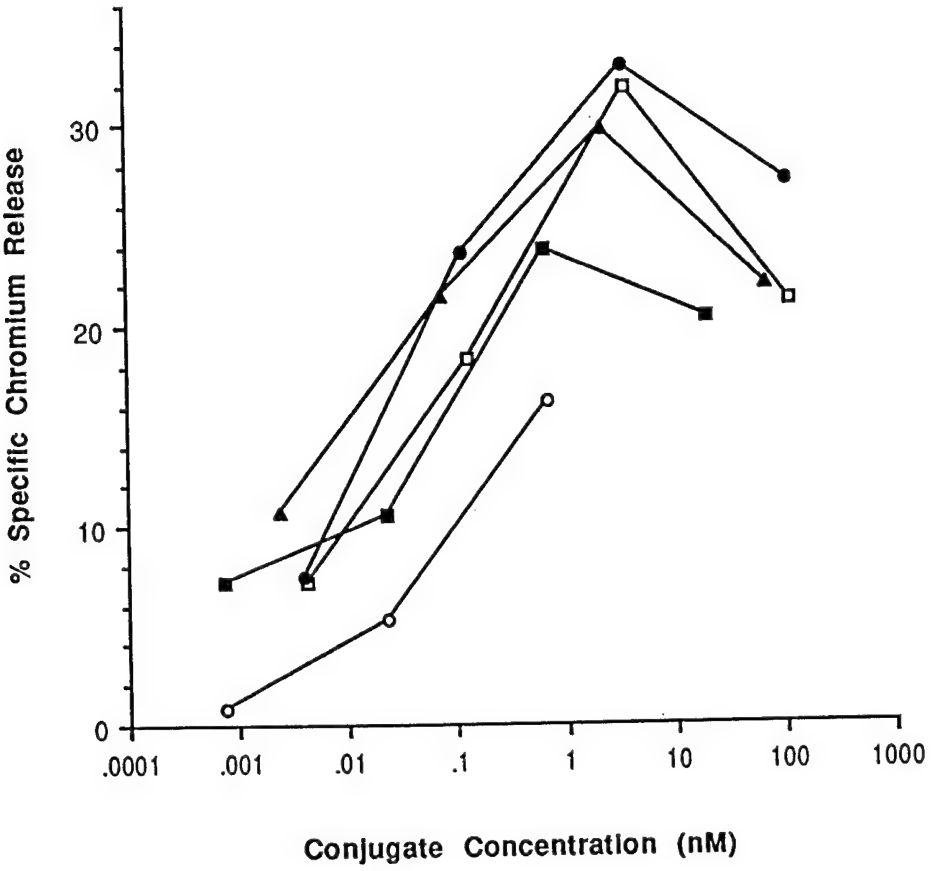
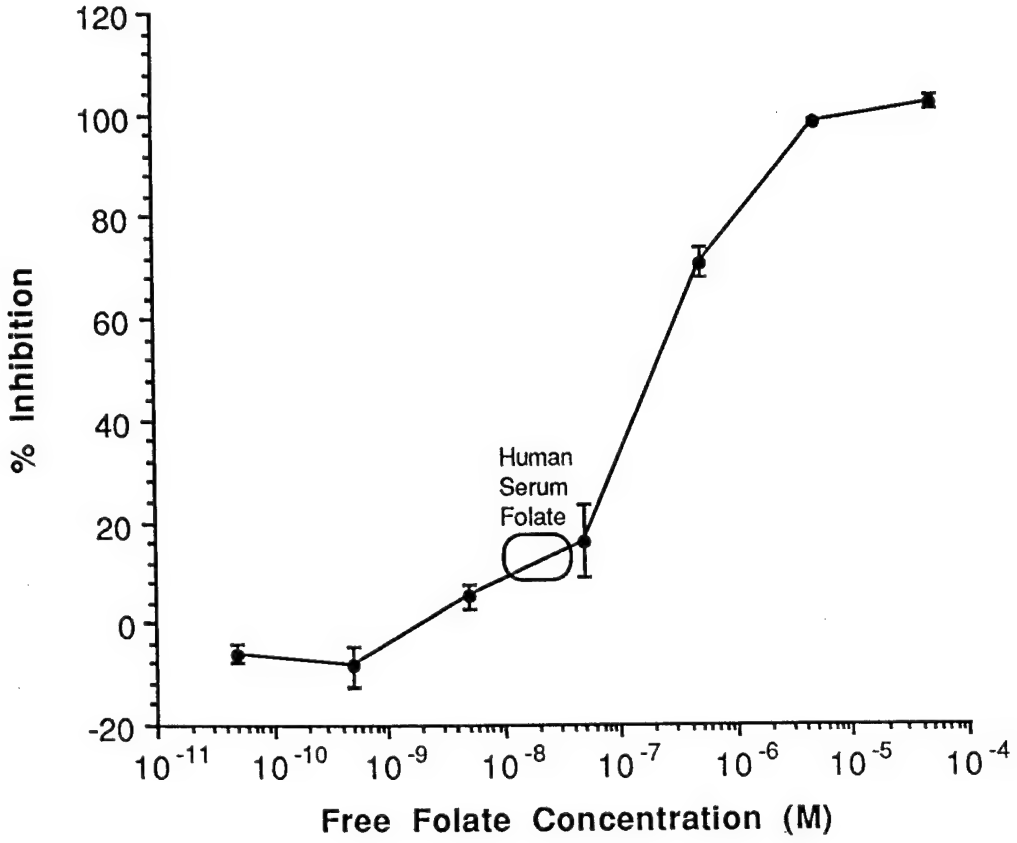
20
10
0
.001
.01
.1
1
10
100
1000
Conjugate Concentration (nM)

B


30
20
10
0
% Specific Chromium Release
.001
.01
.1
1
10
100
1000
Conjugate Concentration (nM)


% Specific Chromium Release
70
60
50
40
30
20
10
0
.00001
.0001
.001
.01
.1
1
10
100
Conjugate Concentration (nM)


% Specific Chromium Release
30
20
10
0
.0001
.001
.01
.1
1
10
100
1000
Conjugate Concentration (nM)


120
100
80
60
40
20
0
-20
% Inhibition
Human
Serum
Folate
10 -11
10 -10
10 -9
10 -8
10 -7
10 -6
10 -5
10 -4
Free Folate Concentration (M)

Lysis of Breast Cancer Cells Mediated by a Bispecific, Single-Chain Anti-erbB-2 Antibody

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Short Title: Bispecific Single-Chain Anti-erbB-2 Antibody

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Abstract

Bispecific antibodies that bind to erbB-2 and CD3 have been shown to mediate the lysis of erb-B2 positive breast cancer cells by cytotoxic T lymphocytes. Various forms of bispecific antibodies have been produced, but the form that will be most effective *in vivo* has yet to be determined. To begin to address this issue, we have engineered a bispecific single-chain antibody ($scFv_2$) to erbB-2 and the mouse T cell receptor from the CTL clone 2C. This TCR has been previously expressed in transgenic mice and we have recently produced immunodeficient mice that express a monoclonal population of CTL with this receptor. In anticipation of targeting transplanted human tumors in these mice, the bispecific $scFv_2$ is characterized in this report. The erbB-2 specific monoclonal antibody 800E6 was cloned and expressed in *E. coli* as a 30kDa single chain Fv (scFv). Binding to erbB-2^+ SKBR-3 cells demonstrated that the affinity of the scFv for erbB-2 was identical to that of 800E6 Fab fragments (20nM). The bispecific $scFv_2$ 1B2/800 was constructed by joining anti-TCR scFv, 1B2, to the 800E6 scFv with a 25 residue linker. The 60kDa 1B2/800 $scFv_2$ bound to erbB-2 and could redirect lysis of erbB-2^+ BT-474 cells by the CTL clone 2C at concentrations of 1μg/ml and above. This antibody can now be tested in comparison with other engineered and conventional bispecific forms in the transgenic mouse model.

Key Words: Bispecific Antibody, erbB-2, Cytotoxic T Lymphocytes

Introduction

Tumor-associated antigens present on the cell surface can serve as potential targets in the specific elimination of tumor cells. One such target is the oncogene product erbB-2 which is overexpressed in approximately 30% of human breast cancers(1). The efficacy of bispecific antibodies in redirecting cytotoxic T cell lysis of erbB-2^+ cells has been demonstrated in studies using anti-erbB-2 hybrid hybridomas and chemically coupled antibody fragments(2-5). Despite some successes of these bispecific antibodies in tumor therapy, the optimum method of preparing clinically useful antibodies has not been resolved. Each method has particular limitations, but in principle the optimal form will be the smallest possible agent with the highest affinities for both the tumor antigen and the T cell receptor. In addition, the yields of the final active agent must be sufficient to allow treatment in humans.

Recombinant technology and the generation of single chain fragments (scFv)(6-9) have led to the construction of single chain bispecific ($scFv_2$) antibodies which may provide a more optimal method for preparing clinically useful reagents(10, 11). Production of the $scFv_2$ in *E.coli* provides a possible solution to problems of heterogeneity and yield, while the smaller size and lack of constant regions may increase tumor penetrance and decrease immunogenicity. Studies have demonstrated that the smaller scFv molecule can penetrate tumors more effectively than intact antibodies or Fab fragments, and they are retained longer in the tumor than in circulation(12). The latter property may also be important in terms of lower toxicity and immunogenicity.

Despite the progress on engineering various forms of bispecific antibodies, there has been little effort toward comparing their *in vivo* effects in an animal model. An ideal model would allow *in vivo* activation and targeting of transplanted human tumors with endogenous CTL. Toward this goal, we recently produced double transgenic mice that have the RAG-1 gene knocked out(13), and the TCR α and β chain genes(14) from the CTL clone 2C (M. M. Gruber and D. M. Kranz, unpublished). These immunodeficient mice express a monoclonal population of CTL that do not recognize various human breast cancer lines.

To begin engineering bispecific antibodies to be tested in this model, the scFv of the anti-TCR antibody 1B2 (15, 16) was linked in this study to a scFv of the anti-erbB-2 antibody 800E6. The 800E6 antibody was previously shown to have a high affinity for the extracellular domain of erbB-2(17). We report here the characterization the 60kDa bispecific $scFv_2$ antibody that targets erbB-2 and mediates CTL 2C lysis of erbB-2 bearing tumor cells.

Materials and Methods

Cell Lines. The BALB.B derived CTL clone 2C, which recognizes the alloantigen L^d(18) and the superantigen SEB(19), was maintained in complete RPMI media [RPMI 1640 (Gibco, Grand Island, NY) containing 5mM HEPES (Sigma, St. Louis, MO), pennicillin, streptomycin, 10% fetal bovine serum (Gibco), 1.3 mM L-glutamine (Gibco), 50μM 2-mercaptoethanol (Sigma)], and 10% supernatant from Con A-stimulated rat spleen cells as previously described(18). 2C cells were stimulated approximately every week with mitomycin C-treated BALB/c spleen cells. Daudi, a human

lymphoma that expresses class II HLA molecules, was maintained in complete RPMI media. Human breast carcinomas were obtained from the ATCC(Rockville, MD). SKBR-3 was maintained in McCoy's 5A medium containing 5mM HEPES, pennicillin, streptomycin, 10% fetal bovine serum, 1.3 mM L-glutamine, 50μM 2-mercaptoethanol. BT-474 was maintained in complete RPMI medium with 10μg/ml bovine insulin (Gibco). 800E6 is a BALB/c derived murine hybridoma which secretes an IgG1 monoclonal antibody that is specific for the erbB-2 transmembrane glycoprotein(17). 800E6 was maintained in complete RPMI medium.

Preparation of Intact Antibodies and Fab Fragments. Intact 800E6 was purified from ascites fluid by ammonium sulfate precipitation followed by DEAE column chromatography. Antibody was precipitated in 50% ammonium sulfate, the pellet resuspended in 50mM K_2PO4, pH7.5, and then extensively dialyzed. A portion of intact antibody was dialyzed into TBS at ~2.2 mg/ml and Fab fragments were generated by digesting the antibody for 30 min in 2 U/ml papain (Sigma) at 37°C. Fab fragments were purified from residual intact antibody by size exclusion chromatography over a Superdex 200 column (Pharmacia, Piscataway, NJ).

Construction and Expression of 800scFv. Total RNA was isolated by the guanidinium thiocyanate-CsCl method. cDNA was generated by using approximately 5μg of the RNA with 35 units of reverse transcriptase (Promega, Madison, WI) and 5μM of oligoDT primer. Antibody V_L and V_H genes were cloned using the polymerase chain reaction (PCR) and degenerate primers for mouse antibodies: V_H: 5'-

AAAGATGCATCCCAGGTGCAGCTGCAG(GC)AGTC(AT)GG(AG)(GC)(GC)G-3'; J_H: 5'-AAATAAGCTTTTGTTCTGAGGAGACGGTGACTGAGGTTCCT(GT)(GC)(AG)CCCCA-3'; V_L: 5'-ATCGGACGTCGTGATGAC(CT)CAGTCTC-3'; J_L: 5'-TGATCCGCGGAGGAACGTTTTATTTCCAGCTTGGTCCC(ACT)(GC)C(AGT)CCGAA-3'. The V_L and V_H genes were inserted into a plasmid containing the 26 amino acid linker 205s(20). The construction included the ompA signal sequence and a 10 residue carboxy terminal tag derived from the *c-myc* protein. To construct the 1B2/800 $scFv_2$, 800E6 scFv was amplified by PCR with a 5' primer that included a Hind III site followed by a 205c'(7)linker gene (primer 5'-ATCTAAGCTTGCTAGCGCAGACGATGCCAAAAAAGACGCGGCGAAAAAAGACGATGCCAAAAAGGACGACGCCAAAAAAGATCTTGACGTCGTGATGACTCAGTCTCACAAATTCA-3') and a 3' primer to the plasmid (5'-GAACAAATCCAGATGGAGTTCTGA-3'). The modified 800E6 scFv gene was inserted into a plasmid containing the 1B2 scFv gene(16) The 1B2 scFv gene consists of the ompA signal sequence, the 1B2 V_L gene, a 42-base pair linker gene [called 212,(19)], the 1B2 V_H gene, and a 30-base pair gene encoding the *c-myc* peptide(21). The modified 800E6 scFv was inserted at the Hind III site between the 1B2 scFv V_H gene and the *c-myc* gene. All primers were synthesized by the Genetic Engineering Facility at the University of Illinois Biotechnology Center. Both 800E6 scFv and 1B2/800 $scFv_2$ were expressed, as previously described(22), using a hybrid O_L/P_R I phage promoter in an *E.coli* strain that contains a

temperature sensitive C_{1857} repressor gene. Induction, solubilization, and purification were performed as previously described(22). Briefly, small scale cultures or fermentations were grown to an A_{600} of 1.0 at 30°C, at which point recombinant protein expression was induced by temperature shift to 42°C for 1 hr. Cells were disrupted by passage through a microfluidizer, centrifuged at low speed to pellet unlysed cells, and the supernatant centrifuged to pellet inclusion bodies. Inclusion bodies were solubilized for 12-24 hours in 6 M guanidine hydrochloride (10 ml guanidine per cell pellet equivalent obtained from 1 liter of culture).

Purification of scFv and $scFv_2$ Fragments. 800E6 scFv was refolded by dialysis at ~1mg/ml against 0.1M Tris, pH 8.0, 2mM EDTA, pH 8.0, 0.4M arginine (TEA buffer) at 4°C. Refolded scFv was centrifuged at 15,000xg to remove precipitate and injected onto a Superdex 200 size exclusion column pre-equilibrated in phosphate buffered saline, pH 7.3. 1B2/800 $scFv_2$ was applied to a Superdex 200 size exclusion column pre-equilibrated in 4M guanidine-HCl, 50mM Tris, pH8.0, 2mM EDTA to remove protein still aggregated in the presence of denaturant. Fractions corresponding to the monomeric peak were adjusted to ~1mg/ml in 4M guanidine buffer and refolded by dialysis in TEA buffer. To isolate monomeric 1B2/800, refolded $scFv_2$ was applied to a Superdex 200 size exclusion column pre-equilibrated in PBS, pH7.3. All columns were run at 0.5ml/min and eluents monitored by absorbance at 280nm.

Binding Assays. To determine the intrinsic affinity of the 800E6 Fab fragments, a saturation binding assay was performed with ^{125}I-labeled Fab fragments. Increasing concentration of labeled 800E6

Fab fragments were added to wells containing $2x10^5$ SKBR-3 to a total volume of 75µl. After a 60 minute incubation on ice, 50µl was layered over 300µl of 20% olive oil, 80% dibutylphthalate in an elongate microcentrifuge tube and flash spun to pellet cells. Tubes were frozen on dry ice/ethanol and cut to isolate the cell pellet from the solution. Fractions were monitored in a γ-counter and a Scatchard plot was generated to determine K_D of the Fab fragments. To detect scFv and $scFv_2$ binding to erbB-2 on the surface of breast cancer cells, a competition assay was performed with ^{125}I-800E6 Fab fragments. Various concentrations of unlabeled 800E6 Fab fragments, scFv, or $scFv_2$ were added, together with a constant amount (200,000 cpm) of ^{125}I-labeled 800E6 Fab fragments, to tubes containing $2x10^5$ SKBR-3 or BT-474 cells in a total volume of 75µl. Fractions were monitored in a γ-counter and inhibition curves were prepared for unlabeled 800E6 Fab fragments, scFv, and $scFv_2$ antibodies.

Cytotoxicity Assays. Target cells were harvested by trypsinization, washed with RPMI medium, and incubated with 50µl of ^{51}Cr (2.5mCi/ml) for 1 hr at 37°C. After washing with RPMI media, cells were added to round bottom wells of a 96-well plate at $2x10^4$ cells/well. 1B2/800 $scFv_2$ was added at various concentrations, and 2C was added at $2x10^5$ cells/well. Assays were incubated for 4 hrs at 37°C in a 5%CO_2 incubator. Supernatants were removed and monitored with a γ-counter. Specific ^{51}Cr-release was determined as follows: %Specific ^{51}Cr release = [(experimental counts - spontaneous counts)/(maximal counts - spontaneous counts)]x100.

Results

Cloning and construction of 800E6 scFv. PCR of cDNA from anti-erbB-2 hybridoma 800E6 was performed using degenerate primers for mouse V_H and V_K. Amplified product of the expected sizes (350-400 bp) were obtained for each set of primers (data not shown). The V_H product was cloned into a vector flanked at the 5' end by the sequence for a 26 amino acid linker, 205s(20) and at the 3' end by the sequence for a 10 residue peptide derived from the *c-myc* protein. This *c-myc* tail was inserted at the carboxy terminus in order to allow detection with the anti-*c-myc* monoclonal antibody, CT14-G4.3(23). The V_K gene was then cloned upstream of the V_H between the ompA signal sequence and the 205s linker (Figure 1A).

The nucleotide and predicted amino acid squences of 800E6 scFv are shown in Figure 1B. Both the V_H and V_L genes contained the typical invariant residues present in Ig domains, including two appropriately spaced cys residues and the GXGT motif within the J regions. The calculated molecular mass of the mature protein is 28,772 daltons.

Expression and Purification of 800E6 scFv. Previous studies from our lab showed that single chain antibodies were expressed in the insoluble fraction of the lysed *E. coli* cell pellet(10, 16, 20). Thus, inclusion body pellets from cells that contained the 800E6 scFv gene were solubilized in guanidine and dialyzed against a Tris-arginine buffer to allow refolding(24). SDS-PAGE analysis showed that approximately 80% of the refolded inclusion body protein migrated at 30kDa (Figure 2). 800E6 scFv was purified further through a non-

denaturing Superdex 200 size exclusion column (see SDS-PAGE gel, Figure 2).

Binding Properties of 800E6 scFv. The intrinsic affinity of the 800E6 Fab fragment was determined by saturation binding of ^{125}I-Fab fragments to SKBR-3. The K_D was determined by Scatchard analysis to be 20nM(data not shown). To determine the relative affinity of 800 scFv for erbB-2, a competition assay was performed with ^{125}I-labeled Fab fragments of 800E6 and unlabeled Fab fragments or HPLC purified scFv. As shown in Figure 3, scFv bound to erbB-2 on SKBR-3 cells at nearly equivalent concentrations as Fab fragments.

Construction of anti-TCR/anti-erbB-2 bispecific scFv$_2$. Bispecific single-chain (scFv$_2$) 1B2/800, with binding domains of both the anti-TCR antibody 1B2 and the anti-erbB-2 antibody 800E6, was constructed as shown in Figure 4. 1B2 is a clonotypic antibody that reacts with the TCR from the BALB.B CTL clone 2C(15). The 1B2 scFv construction contained the V_L and V_H chain genes, intra-antibody linker, 212, and a 10 residue c-*myc* tail at the carboxy terminus. To construct the scFv$_2$, the 800E6 scFv was amplified by PCR using a 5' primer which contained the sequence for the 205c' linker, and a 3' primer that included a HindIII site within the c-*myc* region for cloning into the 1B2 scFv vector. The linker/800E6 scFv fragment was cloned between the 1B2V_H and the *c-myc* tail as shown in Figure 4.

Expression and Purification of anti-TCR/anti-erbB-2 scFv$_2$. Inclusion body pellets from cells that contained the scFv$_2$ gene were solubilized in 6M guanidine. To separate monomeric scFv$_2$ from

proteins which remained aggregated in the presence of guanidine, the solubilized inclusion body was subjected to Superdex 200 size exclusion in 4M guanidine. The 60kDa peak fractions were each refolded by dialysis into Tris-Arginine buffer, and analyzed by SDS-PAGE (data not shown). Fractions containing the 60kDa protein were pooled, dialysed against buffer, and subjected to non-denaturing size exclusion chromatography. The peak that eluted at the approximate size of monomeric $scFv_2$ (~60kDa) migrated as two distinct species under reducing conditions (data not shown) and non-reducing conditions(Figure 5). The second protein may be a contaminant from *E. coli*, or a differentially processed form of the $scFv_2$ (with or with out the signal sequence). Although we have not examined these possibilities directly, we have observed that the binding activity of the HPLC purified 1B2/800 $scFv_2$ (see below) is equal to that of a histidine-tagged version of 1B2/800 (1B2/800-6histidine) that was purified on an immobilized metal affinity column (M. M. Gruber, and D. M. Kranz, unpublished). This suggests that the HPLC purified material contained approximately the same fraction of properly refolded $scFv_2$ as the histidine-tag purified protein.

Binding Properties of anti-TCR/anti-erbB-2 $scFv_2$. To examine binding of the $scFv_2$ to erbB-2 on the surface of human breast cancer cells, a competition assay was performed with ^{125}I-labeled 800E6 Fab fragments. As shown in Figure 6, crude as well as partially purified preparations of $scFv_2$ were able to bind erbB-2 on BT-474 cells. In contrast to the monovalent 800E6 scFv which bound erbB-2 at nearly equimolar concentrations as 800E6 Fab fragments, bispecific antibody preparations were 10 to 25-fold less efficient than

unlabeled Fab fragments. A possible explanation for the observed reduction in binding is that a significant fraction of the $scFv_2$ preparation is not properly folded.

Bispecific Antibody Activity in CTL-Mediated Killing Assays. As the $scFv_2$ had erbB-2 binding activity, it was of interest to examine if the antibody could redirect CTL 2C to lyse target cells. As expected, the L^d alloreactive CTL 2C was unable to lyse human, erbB-2 positive breast carcinoma line BT-474 without the bispecific antibody. However, BT-474 were lysed when 1B2/800 $scFv_2$ was added to the CTL/target cell assay (Table 1). The observed lysis was dependent on the concentration of bispecific antibody (Figure 7) although the maximal lysis obtained with BT-474 cells never reached more than ~25% specific release. The reason for this is unknown. As expected, $scFv_2$ mediated lysis could be inhibited with monospecific 1B2 or 800 intact Ab (Table 1, exp. 1). The lysis required covalent linkage of the two antibody binding domains, as evidenced by the inability of a mixture of 1B2 and 800E6 monospecific antibodies to redirect lysis(Table 1).

Since anti-TCR antibodies also have the potential to suppress T cell responses, we tested whether concentrations of $scFv_2$ that led to lysis were inhibitory in another cytolytic assay. In this case, the human lymphoblastoid line, Daudi, was incubated with the superantigen SEB which is recognized by CTL 2C(23). This recognition is inhibited at 1B2 concentrations that are several fold lower than inhibition of the peptide/L^d ligand recognized by 2C(16). The 1B2/800 $scFv_2$, however, did not inhibit the recognition at concentrations of 25μg/ml (Table 1). Thus, it should be possible to

use concentrations of bispecific antibody that are effective at mediating killing but will not inhibit normal TCR-mediated responses. However, this result also suggests that the 1B2 domain of the $scFv_2$, like the 800E6 domain, is not folded in a large fraction of the protein.

Discussion

Bispecific antibodies that bind to erbB-2 on breast cancer lines have been used *in vitro* and *in vivo* in T cell mediated therapy(3, 4, 25-28). *In vivo* mouse models using bispecific antibodies against human erbB-2^+ xenografts have demonstrated that redirected T cell lysis leads to decreased tumor size and prolonged survival(4). In these studies, both the bispecific agent and *in vitro* activated human T cells were administered to the mice receiving the transplanted tumors. Athough clinical trials have begun to examine the efficacy of the first generation of bispecific antibodies, $F(ab')_2$ fragments, recent advances in genetic engineering have allowed various smaller and potentially less immunogenic forms to be produced. These forms have included non-covalently associated scFv and Fab fragments, and covalently linked $scFv_2$.

To identify the forms that will have the highest probability of success in patients, there are significant issues that need to be addressed in an appropriate animal model. We believe that the animal model should allow the *in vivo* activation of endogenous T cells in order to resemble the situation to be encountered in humans. Transplantation of human tumors into mice has made this scenario impossible because xenotransplants are rejected by immunocompetent mice. To circumvent this problem we have produced an immunocompromised strain of mouse that contains a

monoclonal population of T cells that express the TCR from the CTL 2C. In this report, we have characterized a bispecific single-chain antibody that can redirect lysis by the CTL clone 2C.

The anti-erbB-2 antibody 800E6 and the anti-TCR antibody 1B2 were chosen because they have high affinities. Monomeric 800E6 scFv also retained high affinity after refolding from guanidine solubilized inclusion bodies. Based on these results, greater than 90% of the scFv preparation appeared to be folded properly. In contrast, after refolding the bispecific $scFv_2$ was at least 10-fold less effective than the scFv in inhibiting binding by 800E6 Fab fragments. Although not formally proven, we believe that the presence of other V regions (i.e. 1B2 V_H and V_L) lead to misfolding of the final $scFv_2$ protein. Nevertheless, the $scFv_2$ protein was active at mediating cytolysis of the human tumor cell line BT-474 in the μg/ml(10-100nM) range. This finding indicates that the 25 amino acid residue linker that joins the two scFv has sufficient flexibility to allow CTL and breast cancer cells to be bridged and to activate the CTL.

Based on the 10 to 100-fold greater activity reported for several conventional bispecific antibodies, it is likely that the folding of $scFv_2$ preparations will need to be improved in order to optimize these reagents for *in vivo* use(28, 29). Alternatively, other methods of creating linked scFv domains may be useful(30, 31) and these forms can be prepared using the 1B2 and 800E6 scFv genes in comparison with the $scFv_2$ described here. Finally, the finding that the $scFv_2$ 1B2/800 mediated lysis of erbB-2^+ human tumor cells by the mouse CTL 2C suggests that these agents can now also be compared in the *in vivo* model described above.

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Figure Legend

Figure 1. Schematic of the anti-erbB-2 single-chain antibody 800E6. A. cDNA was generated from the 800E6 hybridoma, and the V_L and V_H genes were amplified by PCR using a set of degenerate primers. The genes were linked by a sequence encoding a 26-amino acid linker called 205s, and the construct was cloned behind the ompA signal sequence and included a carboxyl-terminal peptide from the *c-myc* protein. **B.** Sequence of scFv 800E6 derived from the 800E6 hybridoma, and sequence for *c-myc* tail.

Figure 2. SDS-PAGE analysis of purified 800E6 scFv. Single chain antibodies were prepared as described in Materials and Methods. Crude scFv represents the TEA-dialyzed preparation before size exclusion chromatography, and purified scFv represents fractions from the Superdex 200 column. Molecular mass markers (in kDa) are shown. Proteins were visualized by staining with Coomassie blue.

Figure 3. Binding of scFv to erbB-2 on SKBR-3. Various concentrations of unlabeled 800E6 Fab fragments or scFv were added, together with a constant amount (200,000cpm) of ^{125}I-labeled 800E6 Fab fragments, to wells containing $2x10^5$ SKBR-3 cells. After 1hr on ice, cells were pelleted through a mixture of olive oil and dibutylphthalate, and monitored on a gamma counter. Percent inhibition was determined relative to binding of ^{125}I-labeled 800E6 Fabs in the absence of inhibitor.

Figure 4. Schematic of bispecific single-chain anti-T cell receptor, anti-erbB-2 antibody scFv$_2$. 1B2 V_L and V_H genes, linked by a sequence encoding the 14 amino acid 212 linker (GSTSGSGKSSEGKG), and 800E6 V_L and V_H genes linked by a

sequence encoding the 26 amino acid 205s linker (SSADDAKKDAAKKDDAKKDDAKKDAS) were joined using overlapping primers in the polymerase chain reaction. The monospecific single-chain constructs were linked by a gene that encodes a 25 amino acid linker called 205c' (ASADDAKKDAAKKDDAKKDDAKKDL). The bifunctional single-chain was cloned between the ompA signal sequence (MKKTAIAIAVALAGFATVAQA), and a 10 amino acid peptide from the *c-myc* protein (EQKLISEEDL).

Figure 5. Non-denaturing gel filtration profile and SDS-PAGE analysis of purified scFv$_2$. Crude scFv$_2$ was applied to a Superdex 200 size exclusion chromatography column in 6M guanidine. The 60kDa peak was dialyzed against TEA, pH 8.0 and applied to a Superdex 200 HPLC column in PBS, pH 7.3. Absorbance was montiored at 280nm. The column was calibrated with molecular mass standards, and a 60kDa peak corresponding to the monomeric scFv$_2$ was identified. **Insert. Non-reducing SDS-PAGE of gel filtration HPLC fractions.** All single chain antibodies were prepared as described in the text. HPLC fractions represent 60kDa peak fractions as labeled on the HPLC profile. Molecular mass markers (in kDa) are shown. Proteins were visualized by silver stain technique.

Figure 6. Binding of scFv$_2$ to erbB-2 on BT-474 cells. Various concentrations of 800E6 Fab fragments or scFv$_2$ were added, together with a constant amount (200,000 cpm) of ^{125}I-labeled 800E6 Fab fragments, to wells containing 2x10^5 BT-474 cells. Crude scFv$_2$ represents the TEA dialyzed preparation, before HPLC

purification. $scFv_2$ was purified by passage through Superdex 200 in 6M guanidine. After 1 hr on ice, cells were pelleted through a mixture of olive oil and dibutylphthalate, and monitored on a gamma counter. Percentage inhibition was determined relative to binding of ^{125}I-labeled 800E6 Fabs in the absence of inhibitor.

Figure 7. Bispecific $scFv_2$ 1B2/800 mediates lysis of erbB-2$^+$ human breast cancer cells by CTL 2C. BT-474 cells were incubated with 50μ l of ^{51}Cr (2.5mCi/ml) for 1 hr at 37°C. After washing with PBS, target cells were plated at $2x10^4$ cells/well. The $scFv_2$ was added at various concentrations, and CTL 2C was added at $2x10^5$ cells/well. Plates were incubated for 4 hr at 37°C, then supernatants were removed and monitored with a gamma counter.

Table 1. Tumor cell lysis mediated by $scFv_2$ and CTL clone 2C

Experiment 1	antibody	µg/ml	Inhibitor	% Specific 51Cr Release BT-474
	-	-	-	0
	1B2 Ig + 800E6 Ig	25 + 25	-	4
	$scFv_2$	13	-	22
	$scFv_2$	13	25 µg/ml 1B2 Ig	0
	$scFv_2$	13	25 µg/ml 800E6 Ig	0
	$scFv_2$	13	-	0*

Experiment 2	antibody	µg/ml	% Specific 51Cr Release BT-474
	$scFv_2$	5	20
	$scFv_2$	2.5	21
	$scFv_2$	1.3	18
	$scFv_2$	0.6	6

Experiment 3	antibody	µg/ml	% Specific 51Cr Release Daudi/SEB
	-	-	64
	$scFv_2$	25	61

*In this assay, target cells were added to wells with media and $scFv_2$, without effector cells. In all other wells 2C CTLs were added at an effector to target cell ratio of 10:1.

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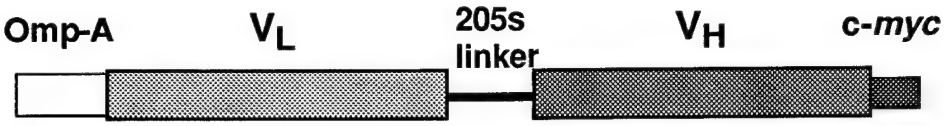
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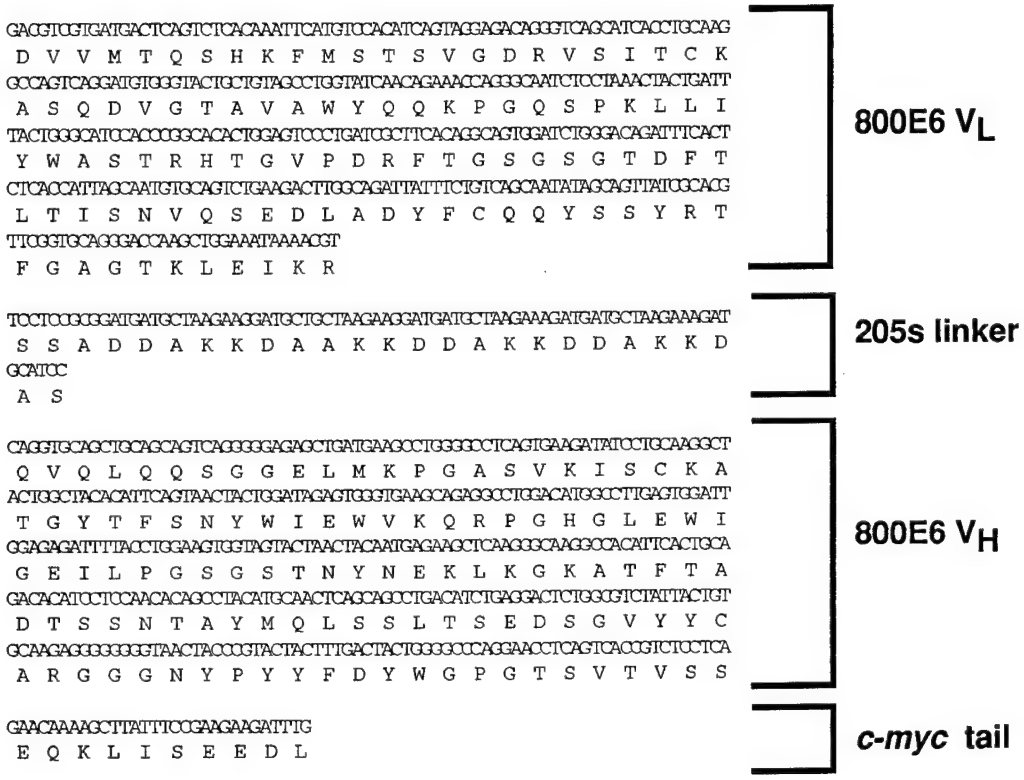
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L T I S N V Q S E D L A D Y F C Q Q Y S S Y R T
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F G A G T K L E I K R
800E6 V_L
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S S A D D A K K D A A K K D D A K K D D A K K D
GCATCC
A S
205s linker
CAGGTGCAGCTGCAGCAGTCAGGGGGAGAGCTGATGAAGCCTGGGGCCTCAGTGAAGATATCCTGCAAGGCT
Q V Q L Q Q S G G E L M K P G A S V K I S C K A
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T G Y T F S N Y W I E W V K Q R P G H G L E W I
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G E I L P G S G S T N Y N E K L K G K A T F T A
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A R G G G N Y P Y Y F D Y W G P G T S V T V S S
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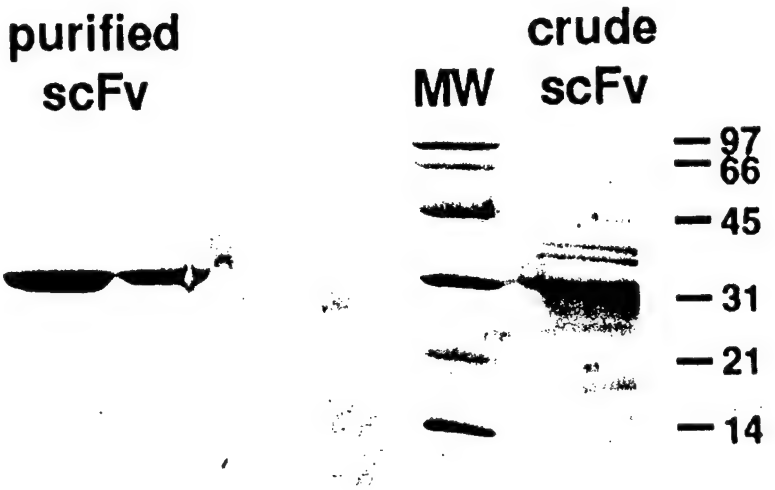
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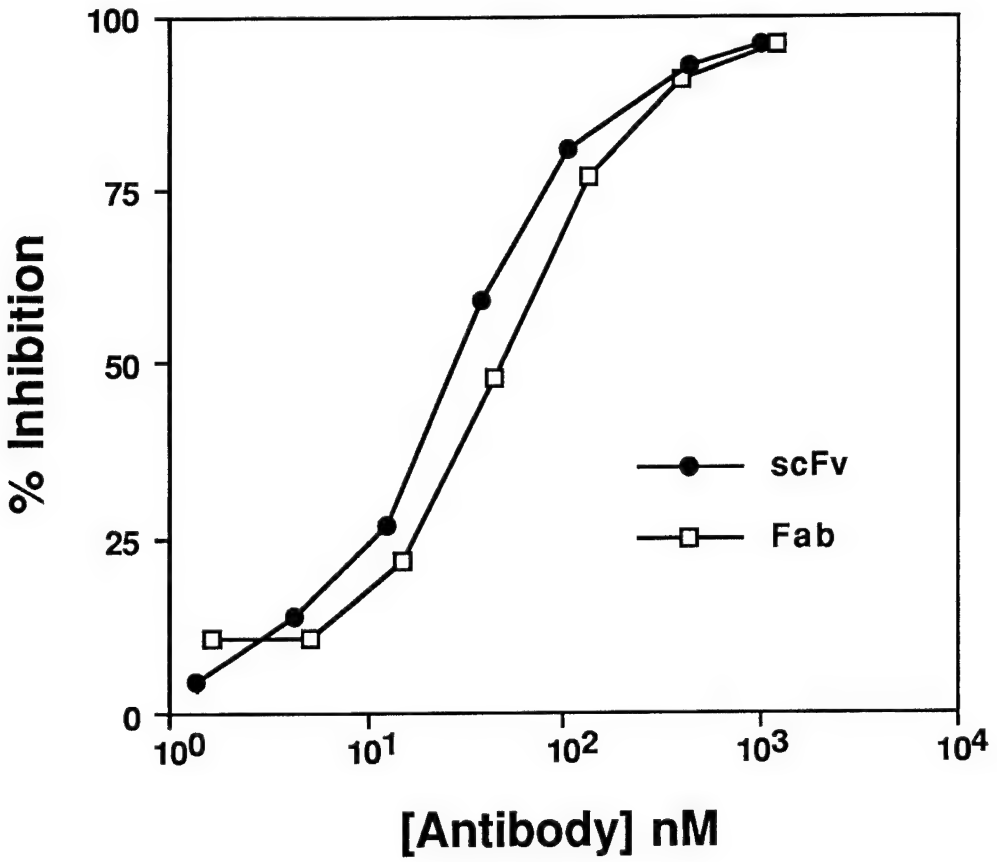
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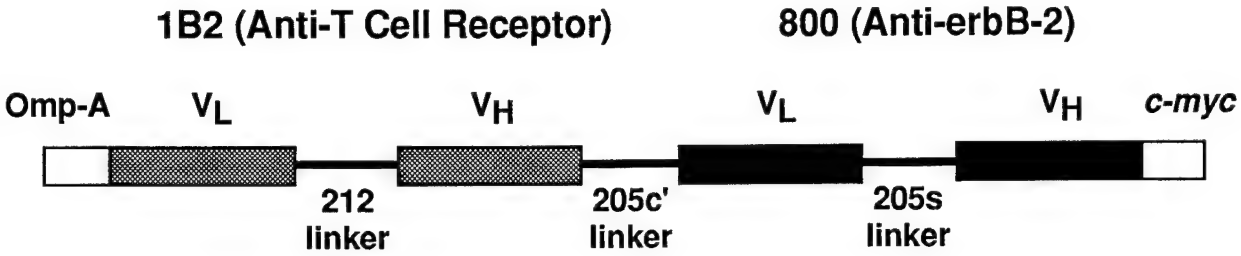
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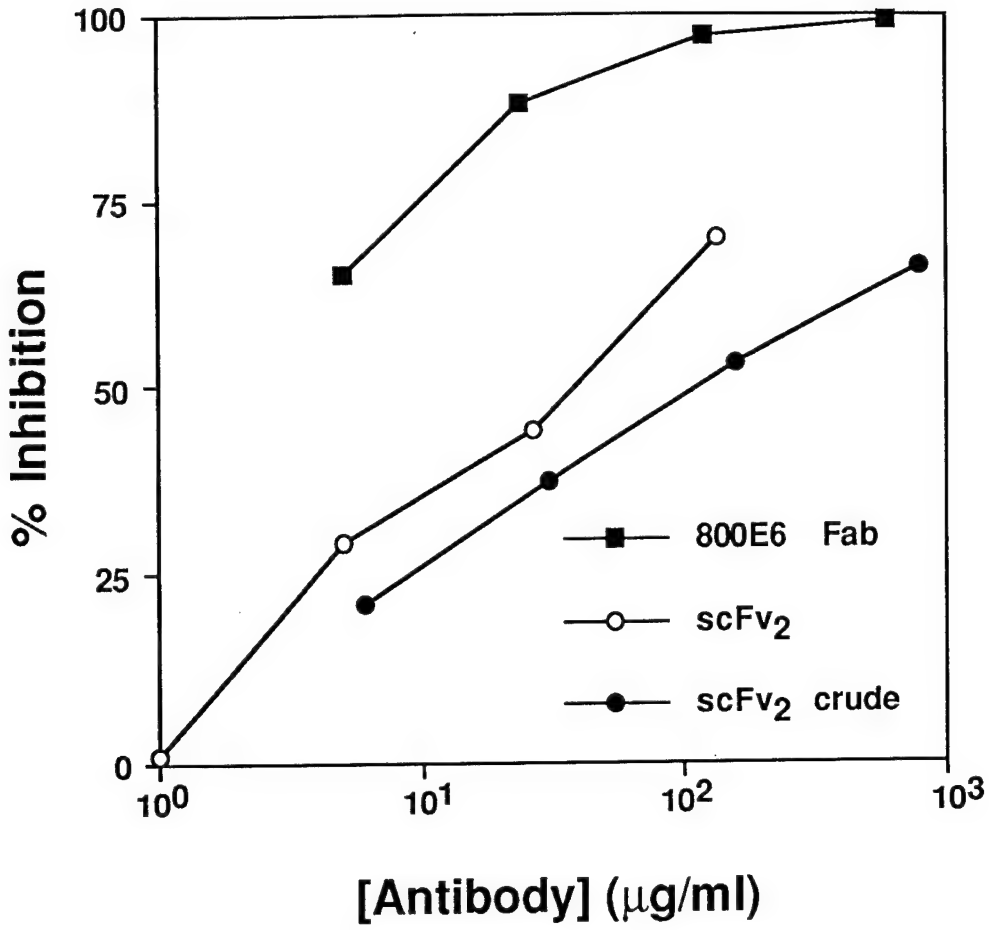
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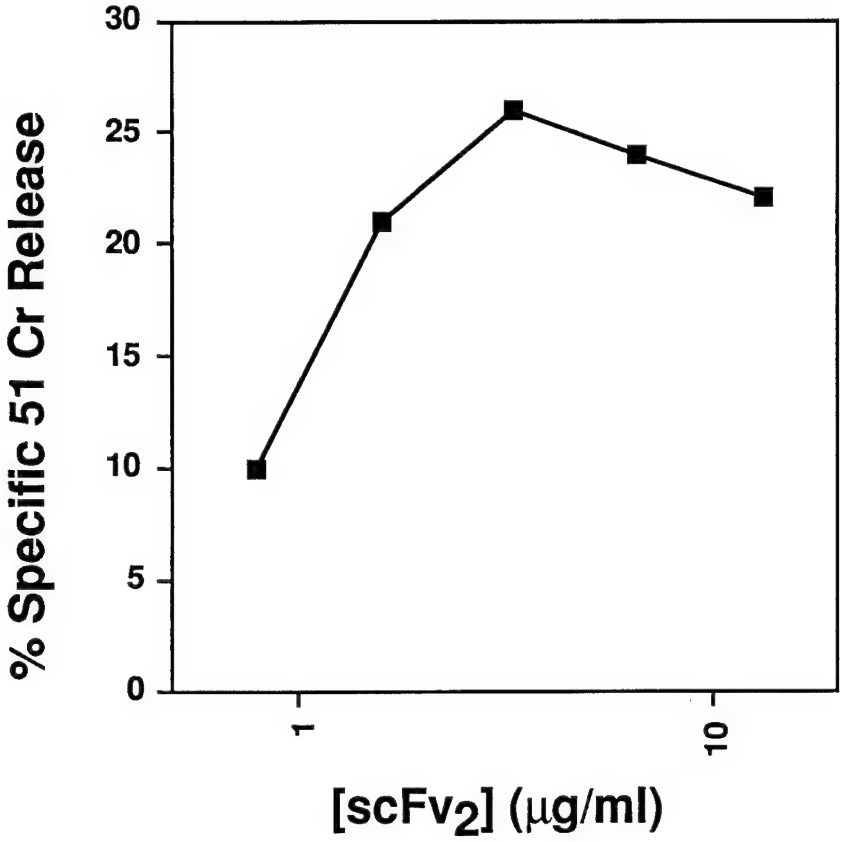
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[scFv2] (μg/ml)

A Combination of IFN-γ and TNF-α Can Increase the Susceptibility of erbB-2 Positive Tumors to CTL-Mediated Lysis

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Running Title: Susceptibility of Breast Cancer to Lysis by CTL

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Abstract

Although bispecific antibodies to erbB-2 have been shown to redirect cell mediated cytolysis of erbB-2 positive tumor cells, there is evidence that some tumor cells are resistant to immune cytotoxic mechanisms. In this report, the susceptibility of erbB-2$^+$ breast cancer lines to lysis mediated by a bispecific antibody and the mouse cytotoxic T cell clone (CTL) 2C was evaluated. The bispecific single chain antibody ($scFv_2$), 1B2/4420, which recognizes the T cell receptor on CTL 2C and the hapten fluorescein was used in order to eliminate the effect of erbB-2 density on recognition. Thus, covalent attachment of equivalent amounts of fluorescein to different tumor cells allowed this bispecific agent to be used to examine the intrinsic susceptibility of tumor cells to T cell mediated lysis. Among the panel of tumor lines tested, the absolute level of erbB-2 was not correlated with susceptibility. However, the erbB-2$^+$ cell line SKBR-3 was found to be highly resistant to lysis by CTL 2C. This observation prompted us to screen for agents that might increase the susceptibility of SKBR-3 to lysis. A combination of IFN-γ and TNF-α, but not either agent alone, was effective at increasing susceptibility. This cytokine induced effect was not directly correlated with increased levels of ICAM-1 or Fas, and cytokine treatment did not alter the surface expression of erbB-2. These results indicate that some erbB-2$^+$ tumors may be resistant to the lytic mechanisms of cytotoxic T lymphocytes. Strategies that lead to localized expression of particular cytokine combinations, such as IFN-γ and TNF-α, have potential in reducing this resistance.

Key Words: Bispecific Antibodies, erbB-2, Cytokines, IFN-γ, TNF-α

Introduction

Human breast cancers that overexpress the oncogene product erbB-2 are usually aggressive tumors that lead to poor overall prognosis. Perhaps related to this observation are the results of several reports that suggest erbB-2 expression is correlated with resistance to lysis by TNF-α or lymphokine activated killer cells [9, 13, 20].

The presence of erbB-2 on the cell surface has provided a potential target for the elimination of these tumor cells using various antibody agents(reviewed in [4]). Bispecific antibodies have been used to effectively target some erbB-2$^+$ breast cancer cell lines for lysis by cytotoxic T lymphocytes. However, it is not clear if tumor cell lines which overexpress erbB-2 are equally susceptible to CTL-mediated lysis. If they are not, resistance may lead to difficulty in eliminating such tumors using immunotherapeutic strategies that involve bispecific antibodies to erbB-2 and the T cell receptor or that involve immunogenic peptides that elicit CTL activity.

In this study, the bispecific single chain antibody ($scFv_2$), 1B2/4420, was used to evaluate the susceptibility of different erbB-2$^+$ breast cancer cell lines to cytotoxic T cell mediated lysis, and to screen agents which may increase susceptibility. The $scFv_2$ 1B2/4420 contains the V_H and V_L regions of an anti-T cell receptor antibody, 1B2, and the V_H and V_L regions of an anti-fluorescein antibody, 4420 [11]. Covalent attachment of equivalent amounts of fluorescein to different tumor cells allowed this bispecific agent to be used in examining the intrinsic susceptibility of the tumor cells to cytotoxic T cell mediated lysis. Using this approach, the erbB-2$^+$ cell line SKBR-3 was found to be highly resistant to lysis by the cytotoxic T cell clone 2C. This is in contrast to several other human and mouse tumor cell lines that were efficiently lysed, even when the antigen fluorescein was present at lower densities. The erbB-2$^+$ cell lines SKBR-3,

MCF-7, T-47D, MDA-MB-453, MDA-MB-468, SKOV-3, and BT-474 were found to be susceptible to CTL lysis to varying degrees. Susceptibility was not absolutely correlated with the relative levels of erbB-2 expressed on each cell line.

There is evidence that the cytokines TNF-α and/or IFN-γ can increase susceptibility of some tumor cell lines to lymphokine activated killer cells and tumor infiltrating lymphocytes [1, 8, 26]. In addition, there has been speculation that in vivo administration of IFN-γ and TNF-α may increase tumor susceptibility to $CD8^+$ TIL[2]. To determine whether these cytokines could increase susceptibility of erbB-2^+ tumors to CTL lysis, cytokine treated erbB-2^+ lines were labeled with FITC and assayed for lysis by the $scFv_2$ and CTL clone 2C. A combination of IFN-γ and TNF-α, but not either cytokine alone, was effective in increasing susceptibility to lysis (e.g. cytolysis of SKBR-3 increased on average from <5% to >30%). This combination was also able to increase the susceptibility of MCF-7, T-47D, and MDA-MB-361, but not MDA-MB-453, BT-474, or SKOV-3. The increase in susceptibility to lysis required incubation of tumor cells with the cytokines for 24 to 48 hours.

IFN-γ and TNF-α may increase tumor cell susceptibility through a variety of mechanisms. Reports suggest that IFN-γ and TNF-α decrease erbB-2 levels, rendering cells susceptible to immune cytotoxic mechanisms [19]. IFN-γ and/or TNF-α have also been reported to increase cell surface densities of adhesion molecules [1, 21], MHC class I [14]and II [23], and tumor antigen [16]. Another possible mechanism of cytokine action was an increase in Fas, which could lead to enhanced interaction with Fas ligand (FasL) on the CTL [22], (reviewed in [15]). To examine several of these possibilities, the levels of erbB-2, ICAM-1, and Fas were examined after treatment with IFN-γ and TNF-α, alone or in combination. Treatment with IFN-γ alone led to an increase in ICAM-1 and Fas,

but not erbB-2. However IFN-γ alone did not result in an increased susceptibility to lysis. We conclude that some other post-binding mechanism must account for the effects of the combination of IFN-γ and TNF-α.

Materials and Methods

Cell Lines. The BALB.B derived CTL clone 2C, which recognizes the alloantigen L^d [17], was maintained in RPMI media (RPMI 1640, 5mM HEPES, 10% fetal bovine serum, 1.3 mM L-glutamine, 50μM 2-mercaptoethanol, penicillin, streptomycin), and 10% supernatant from Con A-stimulated rat spleen cells as previously described [17]. 2C cells were stimulated approximately every week with mitomycin C-treated BALB/c spleen cells. Daudi, a human lymphoma that expresses class II HLA molecules, was maintained in RPMI media. Human breast and ovarian carcinomas were obtained from the ATCC (Rockville, MD). SKBR-3, and SKOV-3 were maintained in McCoy's 5A medium containing supplement (5mM HEPES, 10% fetal bovine serum, 1.3 mM L-glutamine, 50μM 2-mercaptoethanol, penicillin, streptomycin). T-47D and BT-474 were maintained in RPMI medium with 10μg/ml bovine insulin (GIBCO, Grand Island, NY). MDA-MB-453 and MDA-MB-468 were maintained in Leibovitz's L-15 medium containing supplement. MCF-7 was maintained in minimum essential medium with non-essential amino acids, 1mM sodium pyruvate, 10μg/ml bovine insulin, and supplement. MDA-MB-361 was maintained in DMEM/F12 with supplement. 900F4 hybridoma, kindly provided by P. Natali, was maintained in RPMI medium. (All media was obtained from GIBCO).

Monoclonal Antibodies. 900F4 is a BALB/c derived IgG1 monoclonal antibody that is specific for erbB-2 transmembrane glycoprotein[7]. 900F4 was purified from ascites fluid by ammonium sulfate precipitation followed by DEAE column chromatography. Anti-human ICAM (CD54) antibody was obtained

from PharMingen (SanDiego, CA). Anti-human Fas (CD95) monoclonal antibody UB2 was obtained from Kamiya Biomedical Co. (Thousand Oaks, CA). The single chain bispecific antibody ($scFv_2$) contains the V_H and V_L regions of anti-TCR antibody 1B2 and the V_H and V_L regions of anti-fluorescein antibody 4420. The $scFv_2$ construction was expressed, as previously described [11], using a hybrid O_L/P_R λ phage promoter in an *E.coli* strain that contains a temperature sensitive C_{1857} repressor gene. Induction, solubilization, and purification were performed as previously described[11]. Briefly, small scale cultures or fermentations were grown to an A_{600} of 1.0 at 30°C, at which point recombinant protein expression was induced by temperature shift to 42°C for 1 hr. Cells were disrupted by passage through a microfluidizer, centrifuged at low speed to remove unlysed cells and the supernatant centrifuged to pellet inclusion bodies. Inclusion bodies were solubilized for 12-24 hours in 6M guanidine hydrochloride (10 ml guanidine per inclusion body pellet equivalent obtained from 1 liter of culture) then dialyzed against 0.1M Tris, pH 8.0, 2mM EDTA, pH 8.0, 0.4M arginine (TEA buffer) at 4°C. Fluorescein-specific $scFv_2$ were isolated by affinity chromatography on Sepharose 6B conjugated to 5-aminofluorescein. The anti-fluorescyl $scFv_2$ antibodies were eluted with 50mM $NaCO_3$, pH 11.5. All columns were equilibrated in 10mM Tris, pH 8.0. Fractions were dialyzed against 10mM Tris, or PBS pH 8.0 at 4°C.

Cytokines. Recombinant human IFN-γ and TNF-α were obtained from R&D Systems (Minneapolis, MN). IFN-γ was diluted to 10^6 units/ml in sterile filtered 10mM acetic acid, 0.1%BSA. TNF-α was diluted to 10^6 units/ml in sterile filtered PBS, 0.1%BSA. Aliquots were stored at -80°C.

Cytotoxicity Assays. Target cells were incubated for various times with different concentrations of IFN-γ, TNF-α, or both prior to the cytotoxicity assay. Target cells were harvested by trypsinization, washed with RPMI medium, and

incubated with 50µl of ^{51}Cr (2.5mCi/ml) for 1 hr at 37°C. After washing with PBS, cells were incubated in 1 ml PBS, pH 7.5 containing 100 µM fluorescein isothiocyanate (Molecular Probes, Eugene, OR) for 15 min at 37°C. Target cells were washed in RPMI media and added to round bottom wells of a 96-well plate at $2x10^4$ cells/well. CTL clone 2C was washed with RPMI media prior to addition to wells. IFN-γ, TNF-α, and $scFv_2$ were added directly to the appropriate wells in the cytotoxicity assay. All assays were incubated for 4 hrs at 37°C in a 5% CO_2 incubator. Supernatants were removed and monitored with a γ-counter. Specific ^{51}Cr-release was determined as follows: %Specific ^{51}Cr release = ((experimental counts - spontaneous counts)/(maximal counts - spontaneous counts)) x 100.

Flow Cytometry. Cells were harvested by trypsinization, washed, and resuspended in RPMI media. $5x10^5$ cells were incubated with anti-ICAM antibody(anti-CD54) , anti-erbB-2 antibody 900F4, or RPMI media on ice for 30 min. The cells were washed in RPMI media and incubated with FITC labeled goat anti-mouse IgG (Kirkegaard and Perry, CITY ST) for 30 min. on ice. FITC labeled anti-Fas antibody UB2 was added to $5x10^5$ cells and incubated on ice for 30 min. For cells labeled directly with FITC, $5x10^5$ cells were incubated with 100µM FITC in PBS, pH 7.3 for 15 min. at 37°C. All cells were washed twice in RPMI media, resuspended in PBS, and analyzed on a Coulter EPICS at the University of Illinois Flow Cytometry Facility.

Results

Intrinsic susceptibility of human tumor lines to CTL lysis. To examine the extent of susceptibility to lysis by CTLs, a panel of human tumor lines was assayed for lysis mediated by a bispecific antibody and the CTL clone 2C. To minimize the effects of recognition on susceptibility, target cells were labeled with fluorescein at approximately equivalent levels (Table 3 and data not

shown) and assayed for lysis in the presence of an anti-TCR/ anti-fluorescein bispecific antibody. As expected, the mouse L^d alloreactive CTL 2C was unable to lyse FITC labeled human target cells in the absence of bispecific antibody (data not shown). Addition of the bispecific antibody resulted in CTL-mediated lysis of most of the target lines (Table 1). A wide range of susceptibilities, from 6% to 56% at an E:T ratio of 10:1 was observed for the panel of tumor lines. Although absolute cytolysis values varied between experiments (probably due to variations in killing efficiency of the CTL clone 2C; unpublished observation), the relative susceptibilities of different lines was consistent. The human lymphoma Daudi, a relatively susceptible cell line, was used in each assay for comparison.

Reports have suggested that erbB-2 overexpression is correlated with increased resistance to cellular cytotoxic mechanisms [9, 20]. To determine if susceptibility to CTL lysis was correlated with erbB-2 expression, erbB-2 levels were examined with the anti-erbB-2 antibody 900F4 by flow cytometry. Although the most resistant line SKBR-3 also expressed the highest level of erbB-2, there was not a strict correlation between erbB-2 levels and susceptibility (Figure 1).

IFN-γ and TNF-α increase target cell susceptibility. To determine whether susceptibility to CTL lysis could be increased by treatment with IFN-γ and TNF-α, the relatively resistant line SKBR-3 was incubated for 48 hours with increasing doses of the cytokines alone or in combination. After coupling of fluorescein, cells were assayed with the $scFv_2$ and CTL 2C. Figure 2A illustrates the dose dependent increase in susceptibility of SKBR-3 after treatment with both IFN-γ and TNF-α. Incubation with 1000U each of IFN-γ and TNF-α resulted in maximal increase in susceptibility. This increase was not

seen with either IFN-γ or TNF-α alone (Figure 2A) and it was not dependent on incubation of CTL 2C with cytokines (data not shown).

The kinetics of increased susceptibility were examined using 1000U each of IFN-γ and TNF-α. SKBR-3 was assayed for susceptibility to CTL-mediated lysis after incubation with the cytokines for 10, 24, and 48 hours. Figure 2B shows that the maximal increase in susceptibilty occured after 24 hours.

To investigate cytokine induced increase in susceptibility in other erbB-2$^+$ tumor lines, MCF-7, MDA-MB-453, MDA-MB-468, T-47-D, SKOV-3, BT-474, and MDA-MB-361 were each incubated with 1000U of IFN-γ and TNF-α for 48 hours. ^{51}Cr release of fluorescein labeled targets with $scFv_2$ and CTL 2C resulted in cytokine induced increases in lysis for MCF-7, T-47-D, and MDA-MB-361, but no increase for MDA-MB-453, MDA-MB-468, SKOV-3, and BT-474 (Table 2). The latter tumor lines exhibited the most susceptibility without treatment. Thus, the cytolytic mechanism induced by cytokine treatment in the more resistant lines may already have been operative in the susceptible lines.

Cytokine induced changes in erbB-2, ICAM-1 and Fas are not correlated with increased susceptibility. To begin to study the mechanism responsible for the cytokine induced sensitivity to lysis, we examined several cell surface molecules known to be involved in effector/target cell interactions. Since cytokine induced susceptibility to CTL lysis was seen in some tumor lines but not in others, it was possible to determine relationships between cytokine induced effects on target cells and increased susceptibility. As discussed earlier, the level of overexpression of erbB-2 did not correlate with intrinsic susceptibility to CTL lysis. There are, however, reports that indicate some cytokines may decrease levels of erbB-2 rendering cells more susceptible to cytokine induced effects[19]. To determine whether a cytokine induced

decrease in erbB-2 was correlated with increased susceptibility, erbB-2 levels on IFN-γ and TNF-α treated cells were examined by flow cytometry using anti-erbB-2 antibody 900F4. No significant decrease was seen in any of the lines, indicating that a change in erbB-2 expression was not a likely mechanism of this cytokine action (Table 3).

Increases in ICAM-1 after cytokine treatment have been implicated in IFN-γ and TNF-α induced increases in susceptibility to cellular cytotoxic mechanisms[21]. To determine if IFN-γ and TNF-α affect ICAM-1 levels, and if the effect is correlated with increased susceptibility, erbB-2$^+$ cell lines were incubated with 1000U of cytokines for 48 hours, and examined with an anti-ICAM-1 antibody by flow cytometry. As previously reported[1, 21], incubation with cytokines increased ICAM-1 levels in all cell lines tested (Table 3). However, the increase was not directly correlated with increased susceptibility since ICAM-1 levels were also raised for cells treated with IFN-γ alone (Table 4). As indicated above, IFN-γ treatment alone did not increase susceptibility to lysis.

Interaction between Fas on target cells and the FasL on T cells can lead to apoptosis of the target [22]. To investigate the possibility of cytokine induced Fas elevation on treated targets, cells were incubated with IFN-γ alone, TNF-α alone, or a combination of both for 48 hours. Flow cytometric analysis with FITC-labeled anti-Fas antibody UB2 showed similar results as were obtained for ICAM-1(Table 4). That is, although Fas levels were slightly elevated with IFN-γ and TNF-α, they were also elevated with IFN-γ alone. These results suggest that there exists a mechanism of action separate from ICAM-1 and Fas level elevation, through which IFN-γ and TNF-α together increase target cell susceptibility.

Discussion

The breast cancer cell lines SKBR-3 and MCF-7 were found to be highly resistant to lysis mediated by CTL clone 2C while MDA-MB-453, MDA-MB-468, MDA-MB-361, T-47-D, and SKOV-3 were more susceptible to lysis. Differences in susceptibility were not directly correlated with erbB-2 or ICAM-1 levels on the tumor cell surface. Previous studies have demonstrated a cytokine induced increase in susceptibility of tumor cells to TIL and LAK cell lysis[8, 20, 26]. In this study, we examined if cytokines could similarly increase susceptibility of erbB-2$^+$ tumors to CTL lysis. A combination of IFN-γ and TNF-α was able to increase the susceptibility of erbB-2$^+$ tumors to bispecific antibody mediated CTL lysis. In contrast to results by Lichtenstein et al.[20], treatment with cytokines did not result in a significant effect on the cell surface level of erbB-2.

Previous studies have examined susceptibility of tumor cells to cytolysis in terms of target cell resistance to effector cell binding as well as resistance to post-binding cytolytic mechanisms [9, 26]. Resistance to effector cell binding can involve the down regulation of surface molecules important for adhesion and/or recognition by the effector cell. Resistance to post binding mechanisms are less well characterized but presumably it involves either the pathway that leads to apoptosis (Fas/FasL) or the pathway that leads to membrane destruction by cytolytic granules and perforin.

To examine if enhanced effector cell binding might be a possible mechanism of the cytokine induced increase in susceptibility to CTL lysis, surface levels of several key molecules were measured after cytokine treatment. Although IFN-γ has been reported to increase MHC levels and thus antigen presentation on target cells, the bispecific antibody system used here minimizes the role of MHC/peptide in CTL activation. However, the role of ICAM-1 in antibody redirected cytolysis has been documented[1, 10, 18, 21]. To

determine the role of ICAM-1 expression in resistance to CTL lysis and cytokine induced susceptibility, surface expression of ICAM-1 was measured before and after cytokine treatment. Susceptibility of untreated cells was not correlated with ICAM-1 levels. In contrast to previous reports[9], ICAM-1 levels were also not correlated with overexpression of erbB-2 and were similar to levels expressed on erbB-2$^-$ cells. Although there was a significant increase in ICAM-1 density after treatment with IFN-γ and TNF-α, this increase was similar to that seen with IFN-γ alone, and therefore was not sufficient to explain the synergistic mechanism necessary to effect increased susceptibility to lysis.

Similar results were seen for Fas antigen on the surface of the erbB-2$^+$ cells. Thus, CTL induced apoptosis as a result of enhanced interactions between Fas and FasL did not appear to account for the increase in lysis (ie. surface levels of Fas increased after treatment with IFN-γ alone, as well as with both IFN-γ and TNF-α).

Previous studies examining the susceptibility of erbB-2$^+$ cells to cytolytic post-binding mechanisms have demonstrated resistance to lysis even in the presence of the lectin Concanavalin A, as well as resistance to lytic perforin granules[9]. In apparent contradiction to the report of Fady *et al.* [9] and the results presented here, several studies have shown that erbB-2$^+$ SKBR-3 could be lysed by CTLs [24, 25, 29]. At this time the explanation for these discrepancies is not known. However, one difference between our study and the others, is the source of effector CTL (mouse CTL clone 2C versus polyclonal human CTL lines). If SKBR-3 is in fact resistant to lysis by the perforin pathway, then the Fas/FasL pathway may be the major mechanism by which human CTL act on SKBR. Accordingly, the mouse CTL clone 2C may not have been able to mediate lysis via this pathway, even though Fas is thought to lack species specificity [27] and CTL 2C has been shown to mediate calcium independent

lysis that is characteristic of the Fas pathway (William Soo Hoo and David M. Kranz, unpublished results). Treatment with the combination of IFN-γ and TNFα could presumably have an affect on either the perforin or the Fas/FasL pathway. We are not aware of studies which have directly addressed either of these possibilities.

Reports on the synergism of IFN-γ and TNF-α have frequently studied the direct action of these cytokines on effector cell activation[5, 12]. Discussion of cytokine induced effects on *in vivo* tumor regression have also focused largely on the increase in activity of cytolytic cells [3, 6] or the direct action of the cytokines on tumor cell proliferation [6, 28]. Our results suggest that the tumoricidal effects of IFN-γ and TNF-α treatment may, in the case of some tumors, be due not only to enhancement of the cytolytic ability of effector cells but also to increased susceptibility of the tumor to the cytolytic machinery.

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Table 1. Intrinsic Susceptibility of Human Breast Cancer Lines to Bispecific Antibody Mediated CTL Lysis.

			% Specific ^{51}Cr Release								
Experiment	$scFv_2$ µg/ml	E:T	Daudi	SKBR-3	MDA-MB-453	MCF-7	MDA-MB-468	T-47-D	SKOV-3	BT-474	MDA-MB-361
1	25	10:1	54	8							
2	50	6:1	82		32						
3	50	30:1	64		27						
4	18	6:1	24	1	4	3	14				
5	50	10:1	54	8	36	6	42	20			
6	50	10:1	56	18	45	24		36	38	53	38

A standard 4 hr ^{51}Cr release was performed on cells incubated with 125µCi ^{51}Cr for 1 hr at 37°C. Cells were washed and incubated in 100µM FITC in PBS, pH7.3 for 15 min at 37°C, washed, and plated at $2x10^4$ cells per well. CTL 2C was added at an E:T ratio of 10:1 and incubated at 37°C in a 5% CO_2 incubator. Supernatants were removed and monitored in a γ-counter. Percent specific ^{51}Cr release was calculated as [(experimental cpm-spontaneous cpm)/(maximal cpm - spontaneous cpm)]x100.

Table 2. Effect of Treatment with IFN-γ and TNF-α on Susceptibility of Cell Lines to CTL Lysis.

	Effector:Target	Cell Line	% Specific ^{51}Cr Release	
			untreated	treated
Experiment 1	10:1	Daudi	50	-
		SK-BR-3	1	29
Experiment 2	10:1	Daudi	51	
		SK-BR-3	3	46
Experiment 3	10:1	Daudi	54	56
		SK-BR-3	8	30
		MCF-7	6	15
		MDA-MB-453	36	35
		MDA-MB-468	42	39
		T-47-D	20	46
Experiment 4	10:1	Daudi	56	63
		SK-BR-3	18	28
		MCF-7	24	35
		MDA-MB-453	45	40
		T-47-D	36	54
		SKOV-3	38	38
		BT-474	53	39
		MDA-MB-361	38	45

Cells were incubated for 48 hours in 1000U/ml IFN-γ and TNF-α, or RPMI medium a 5% CO_2 incubator at 37°C. Cells were harvested by trypsinization, washed in RPMI media and incubated with 125μCi ^{51}Cr for 1 hr at 37°C. Cells were washed and incubated in 100mM FITC in PBS, pH7.3 for 15 min at 37°C, and plated at $2x10^4$ cells per well. CTL 2C was added at an E:T ratio of 10:1 and incubated at 37°C in a 5% CO_2 incubator. Supernatants were removed and monitored in a γ-counter. %Specific ^{51}Cr Release=[(experimental cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)]x100.

Table 3. Effect of IFN-γ and TNF-α on ICAM-1 and erbB-2 levels.

Cell Line	IFN-γ/TNF-α	erbB-2 (MFU)	ICAM-1 (MFU)	FITC (MFU)	% Lysis
SKBR	-	188	115	207	8
SKBR	+	185	189	198	30
MCF-7	-	84	135	204	6
MCF-7	+	79	194	196	15
MDA-MB-453	-	142	109	179	36
MDA-MB-453	+	129	180	182	35
T-47-D	-	96	91	185	20
T-47-D	+	97	172	185	46
Daudi	-	11	78	156	54
Daudi	+	8	94	157	56

Cells were incubated for 48 hours in 1000U/ml IFN-γ and TNF-α or RPMI media in a 5% CO_2 incubator at 37°C. Cells were harvested by trypsinization, washed in RPMI media and split for ^{51}Cr release and flow cytometry. For flow cytometric analysis, $5x10^5$ cells were incubated with anti-ICAM-1, anti-erbB-2 (900F4), or RPMI media 30 min on ice. Cells were washed and labeled with FITC labeled goat anti-mouse IgG for 30 min on ice. Alternaltively, cells were directly labeled with 100μM FITC in PBS, pH7.3 for 15 min at 37°C. All cells were washed twice, resuspended in PBS, and analyzed on a Coulter EPICS. Data shown in mean fluorescence units (MFU). A standard 4 hr ^{51}Cr release was performed on cells incubated with 125μCi ^{51}Cr for 1 hr at 37°C. Cells were washed and incubated in 100μM FITC in PBS, pH7.3 for 15 min at 37°C, and plated at $2x10^4$ cells per well. CTL 2C was added at an E:T ratio of 10:1 and incubated at 37°C in a 5% CO_2 incubator. Supernatants were removed and monitored in a γ-counter. %lysis=[(experimental cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)]x100.

Table 4. Effect of IFN-γ and/or TNF-α on ICAM-1 and Fas levels.

Cell Line	Treatment	ICAM-1 (MFU)	Fas (MFU)
SKBR	-	117	57
SKBR	IFN-γ	273	84
SKBR	TNF-α	235	63
SKBR	IFN-γ/TNF-α	287	86
MCF-7	-	129	48
MCF-7	IFN-γ	176	61
MCF-7	TNF-α	146	52
MCF-7	IFN-γ/TNF-α	212	69

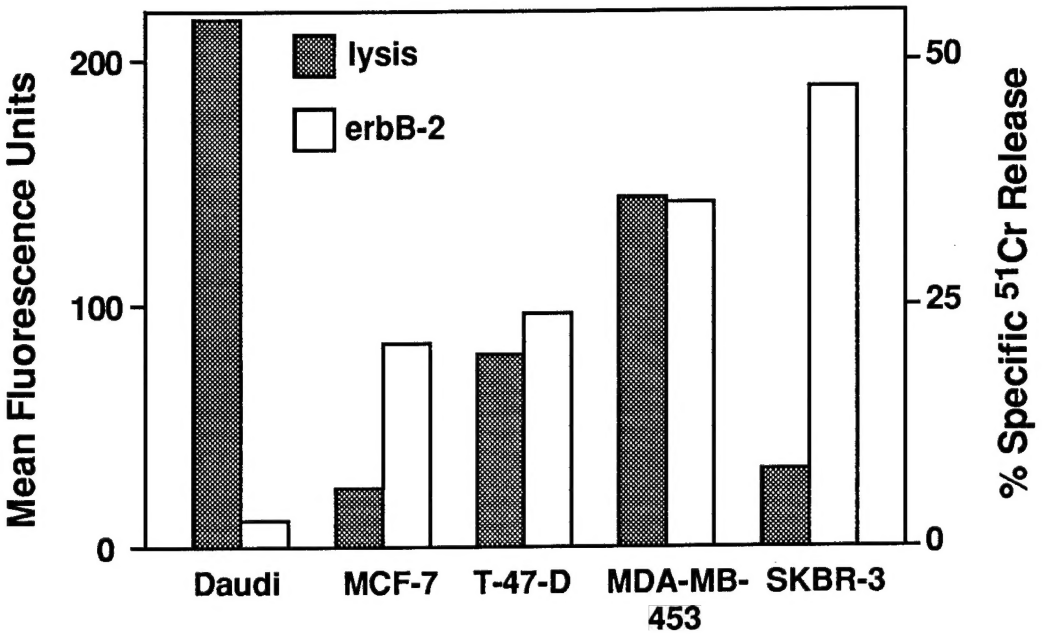
Cells were incubated for 48 hours in 1000U/ml IFN-γ, TNF-α or RPMI media in a 5% CO_2 incubator at 37°C. $5x10^5$ cells were harvested by trypsinization, washed in RPMI media and incubated with anti-ICAM-1, anti-Fas, or RPMI media 30 min on ice. Cells were washed and labeled with FITC labeled goat anti-mouse IgG for 30 min on ice. All cells were washed twice, resuspended in PBS, and analyzed on a Coulter EPICS. Data shown in mean fluorescence units (MFU).

Figure Legend

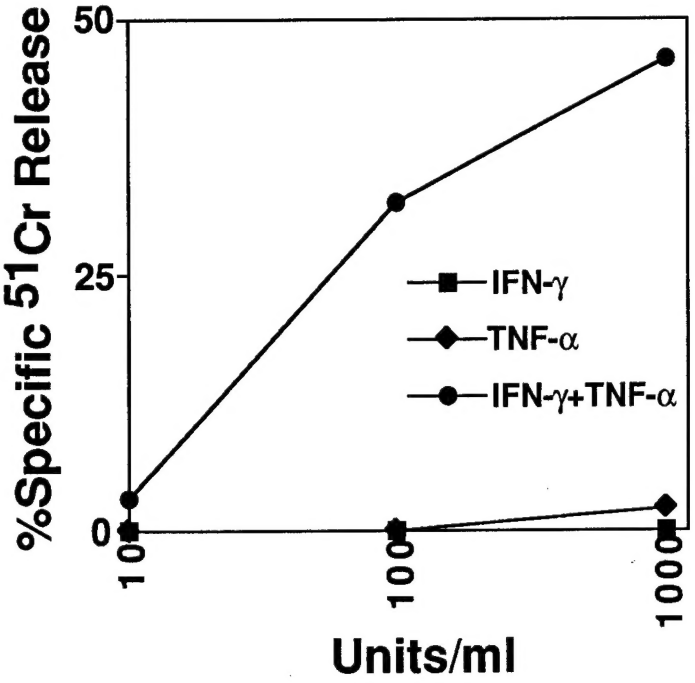
Fig 1. Susceptibility of human breast cancer lines is not correlated to level of overexpression of erbB-2. Cells were harvested by trypsinization, washed in RPMI media and split for ^{51}Cr release and flow cytometry. For flow cytometric analysis, $5x10^5$ cells were incubated with anti-erbB-2 (900F4), or RPMI media 30 min. on ice. Cells were washed and labeled with FITC labeled goat anti-mouse IgG for 30 min on ice. Alternatively, cells were directly labeled with 100μM FITC in PBS, pH7.3 for 15 min at 37°C. All cells were washed twice, resuspended in PBS, and analyzed on a Coulter EPICS. A standard 4 hr ^{51}Cr release was performed on cells incubated with 125μCi ^{51}Cr for 1 hr at 37°C. Cells were washed and incubated in 100μM FITC in PBS, pH7.3 for 15 min at 37°C, and plated at $2x10^4$ cells per well. CTL 2C was added at an E:T ratio of 10:1 and incubated at 37°C in a 5% CO_2 incubator. Supernatants were removed and monitored in a γ-counter. %Specific ^{51}Cr Release = [(experimental cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)]x100.

Fig 2. A. Susceptibility of SKBR-3 to CTL mediated cytolysis is increased by pretreatment with increasing doses of IFN-γ or TNF-α but not by IFN-γ or TNF-α alone. SKBR-3 were incubated for 48 hours in 0, 10, 100, or 1000U/ml IFN-γ, TNF-α, or a combination of both in a 5%CO_2 incubator at 37°C. Cells were harvested by treatment with trypsin-EDTA, washed with RPMI media and incubated with 50μl of ^{51}Cr (2.5mCi/ml) for 1 hr at 37°C. Target cells were then incubated in 1ml 100μM FITC for 15 min at 37°C, washed in RPMI media, and plated at $2x10^4$ cells/well. 2C cells and $scFv_2$ were added directly to the CTL/target assay. All assays were performed at an E:T ratio of 10:1, incubated 4 hrs at 37°C, and supernatants removed and monitored by γ-counter. **B. Preincubation of SKBR-3 with 1000 units/ml**

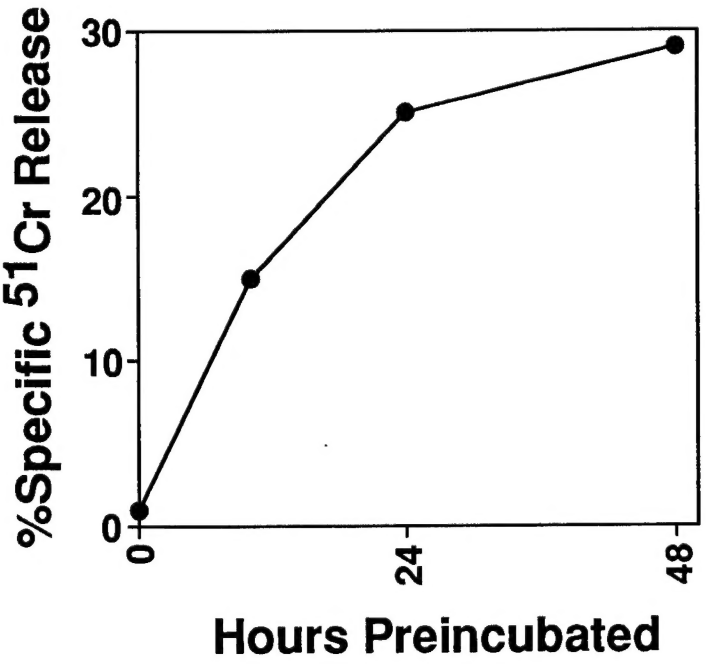
each of IFN-γ and TNF-α increases susceptibility to CTL mediated lysis in a time dependent manner. SKBR-3 were incubated in 1000U/ml IFN-γ and TNF-α for 0, 10, 24, or 48 hours in 5%CO_2 incubator at 37°C. Cells were harvested by treatment with trypsin-EDTA washed with RPMI media and incubated with 50μl of ^{51}Cr (2.5mCi/ml) for 1 hr at 37°C. Target cells were then incubated in 1ml 100μM FITC for 15min at 37°C, washed in RPMI media, and plated at $2x10^4$ cells/well. 2C cells and $scFv_2$ were added directly to the CTL/target assay. All assays were performed at an E:T ratio of 10:1, incubated 4 hrs at 37°C, and supernatants removed and monitored by γ-counter.


Mean Fluorescence Units
200
100
0
lysis
erbB-2
50
25
0
% Specific 51Cr Release
Daudi
MCF-7
T-47-D
MDA-MB-453
SKBR-3

A


50
25
0
%Specific 51Cr Release
IFN-γ
TNF-α
IFN-γ+TNF-α
10
100
1000
Units/ml

B


30
20
10
0
%Specific 51Cr Release
0
24
48
Hours Preincubated